Human Vascular Endothelium from Induced Pluripotent Stem Cells

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Abstract

The vascular endothelium is a dynamic cellular interface that displays a unique phenotypic plasticity. This plasticity is critical for vascular function and when dysregulated is pathogenic in several diseases. The development of new human endothelial genotype-phenotype studies, personalized vascular medicine efforts and cell based regenerative therapies are limited by the unavailability of patient-specific endothelial cells. Induced pluripotent stem cells (iPSC) offer great promise as a new personalized source of endothelium; however, the reproducibility, fidelity and functionality of iPSC-derived endothelial cells remains poorly understood.

In my dissertation research, I have generated vascular endothelium from human iPSC and systematically evaluated their potential for endothelia-specific functionality in order to begin establishing iPSC as a new cellular platform for studying endothelial cell biology. I have reproducibly differentiated iPSC into vascular endothelium exhibiting the functional phenotypic plasticity of mature primary cultured vascular endothelium. These endothelial cells respond to diverse pro-inflammatory stimuli, adopting an activated phenotype including leukocyte adhesion molecule expression, cytokine secretion and support of leukocyte transmigration. They maintain dynamic barrier properties responsive to multiple vascular permeability factors. Importantly, to demonstrate that iPSC-derived endothelial cells are capable of acquiring pathophysiologically-relevant phenotypes critical for modeling human vascular disease, I probed the response of these cells to biomechanical or pharmacological stimuli.

The endothelium derived from iPSC is unique for its lack of an anatomical site of origin and lack of exposure to blood flow. I observed that distinct endothelial cell subpopulations expressing arterial or venous endothelial cell markers are created by differentiating iPSC. I also demonstrated that while some molecular aspects of these identities are specified by genetic programs initially, the endothelial identity is plastic and also can be modulated by biomechanical forces. These results suggest iPSC can yield arterial

and venous endothelium in the absence of flow; but the fate of iPSC endothelial cells in culture is also sensitive to the *de novo* application of biomechanical forces.

My results demonstrate iPSC-derived vascular endothelium possesses a repertoire of functional phenotypic plasticity, and is amenable to cell-based assays probing the endothelial contributions to inflammatory and cardiovascular diseases.

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Acknowledgements

As the saying goes, it takes a village to raise a graduate student, and so it is no different in my case. I am fortunate to have had phenomenal family, friends, mentors and collaborators who deserve special acknowledgement for their direct and indirect contributions to this dissertation, my graduate education, and my constitution.

Principally, I have overwhelming thanks and gratitude to my parents, Jill and Bill Adams, who have overseen my education from first to twenty-third grade. Their support and advocacy have been integral to the success of my graduate education and beyond. I also would like to thank my sister, Kristen Adams, and her fiancé, Craig Scatola, for their support as amidst these Harvard environs, I have tried to maintain a modest foothold in the real world. I cannot imagine a better person with whom to share my successes and disappointments than my wife and eternal collaborator, Lisa Adams. It is based on these relationships that I have always kept sound perspective about life's priorities. I have been lucky to have many true friends Mike Piekny, Gregory C. Zaffiro, Matt Hornick, Megan O'Grady, Borna Dabiri without whom graduate school would have been all work and no play.

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Though this dissertation principally concerns the generation and characterization of a new source of human vascular endothelium, a significant component of this involved juxtaposition with primary human endothelium harvested from human umbilical cord veins. For this, I have been fortunate to enjoy the support of the Cell Biology Core of the Center for Excellence and Vascular Biology at Brigham and Women's Hospital where I have had the pleasure of working with Vannessa Davis and especially through many diverse conversations regarding the liberal arts, Kay Case.

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1 | Introduction to the study of the vascular endothelium

1.1 | Human vascular endothelium and its study *in vitro*

The vascular endothelium is the single-cell layer lining blood vessels that forms an interface between circulating blood and other tissues. Endothelial cells constitute a multifunctional interface that displays a striking phenotypic plasticity necessary for maintaining vascular homeostasis. In this context, the vascular endothelium is critical to initiate an inflammatory response, trigger thrombosis, regulate vasomotor tone and control vascular permeability. Dysfunction of the endothelium plays a significant pathogenic role in cardiovascular diseases, namely, atherosclerosis and its consequences heart attacks and strokes. In addition, genetic mutations in proteins expressed by endothelial cells lead to multiple types of vascular malformations[1,2]. Today, studies at the genetic and molecular level of human endothelium have been limited by the availability of relevant tissue derived from cadaveric, discarded surgical or umbilical vasculature sources.

The cellular layer lining blood and lymph vessels was first given the name endothelium by Wilhelm His in 1865 as he was describing mesodermal development in the chick embryo[3]. During the century that followed, the structure and function of the vascular endothelium was studied primarily through histological and ultrastructural methodologies applied to various model organisms or pathological or cadaveric human tissue. In the early 1970s, the process of isolation of human umbilical cord vein endothelial cells was developed and, critically, the conditions necessary to maintain human endothelial cell growth *in vitro* were defined[4,5]. This foundational work allowed extensive controlled *in vitro* studies which over the last 30 years have defined the scientific and medical community's understanding of the vascular endothelium as fulfilling the diverse functional roles critical for vascular homeostasis mentioned above.

In the decade following the isolation and culture of primary human endothelial cells, advances in mammalian embryology permitted the isolation and *in vitro* culture of mouse embryonic stem cells[6,7]. These stem cells exhibited the pluripotency of the developing embryo such that they can differentiation into all cell types of the organism. As these differentiation processes were studied, they were soon harnessed to permit *in vitro* differentiation of murine embryonic stem cells into mature cell types including vascular endothelium[8]. Almost two decades after murine stem cell lines were generated, human embryonic stem cells were isolated from discarded human embryos created by *in vitro* fertilization[9] and within a few years were able to be experimentally manipulated to produce human vascular endothelium through *in vitro* differentiation[10]. Embryonic stem cells offered practical experimental convenience: the ability to generate vascular isogenic endothelial cells from pluripotent progenitors on demand without the often tedious and inefficient necessity of isolating endothelial cells from heterogeneous tissue samples and the even requirement of procuring discarded tissues in the first place. However, the challenge that embryonic stem cell derived endothelium presents is the uncertainty of the fidelity of *in vitro* stem cell derived tissues to their primary cell analogs in addition to more practical concerns such as reproducibility, cost and time.

Beyond the generation of human vascular endothelial cells to study endothelial cell biology, the genetic revolution at the end of last century created demand for endothelial cells with particular genetic backgrounds to study the functional implications of various disease-associated mutations or polymorphisms in genotype-phenotype studies. It is possible for stem cell lines to be generated from human embryonic stem cells with approximately half of a patient's particular genetic background. This technology is technically cumbersome, expensive and necessitates the ethically controversial creation and destruction of fertilized human embryos, all of which limit the proliferation of personalized stem cell lines and hence personalized vascular endothelium. Further, cell lines incompletely representing a desired genetic background may not aid in genotype-phenotype studies nor constitute a new cellular source for regenerative medicine. Alternatively, it is theoretically possible to crease pluripotent stem cell lines with

complete genetic fidelity to a donor via cloning or somatic cell nuclear transfer, though this has yet to be accomplished in humans.

Recently, the discovery of cellular reprograming as a technology to generate induced pluripotent stem cells (iPSC) offers a potential solution to the challenge of limited cardiovascular cell sources. In this approach, human adult somatic cells, commonly dermal fibroblasts or hematopoietic cells, are de-differentiated into pluripotent stem cells offering comparable function to human embryonic stem cells in their ability to develop differentiated progeny from all developmental lineages in the organism. Human induced pluripotent stem cells were originally derived by retroviral mediated overexpression of four transcription factors, *Oct4*, *Sox2*, *Klf4* and *c-myc*, observed to be present and essential in human embryonic stem cells[11]. However, since this seminal discovery, numerous alternative strategies for derivation have been discovered and validated, some involving integration of exogenous factors into the genome and some without any genome modification, as reviewed elsewhere[12]. The methodology to achieve reprogramming of adult human somatic cells to pluripotency will no doubt continue to be a subject of great academic and commercial interest with efficiency, speed and reproducibility to improve. This technology enables the scientist to choose the particular patient source and hence corresponding genetic background for further study or screening. This patient sample is induced to pluripotency, and subsequently directed to differentiate into the desired cell type. Significantly, these human iPSC are infinitely expandable in their pluripotent form, are generated from minimally-invasive tissue sampling and lack the ethical concerns surrounding manipulation of the human embryo as in embryonic stem cell derivation. It is believed that as iPSC are able to be generated easily from patients, they will constitute a valuable resource for understanding the pathological molecular mechanisms underlying genetic disorders, provide substrate for novel drug discovery and enable various regenerative medicine technologies as a cell based therapy.

In order to be useful for modeling cardiovascular disease and participating in the drug discovery process, iPSC need to be differentiated into cell types relevant for various cardiovascular diseases, namely, endothelium, smooth muscle, cardiac myocytes, fibroblasts as well as hematopoietic lineage cell

types known to participate in vascular disease pathogenesis such as monocytes, lymphocytes and thrombocytes. There has been some progress in generating such cardiovascular disease relevant cell types. In general, this was accomplished either through the formation of differentiating heterogeneous three-dimensional embryoid bodies from which a cell type may be isolated, as exemplified within **Figure 1.1**, or though addition of various factors to encourage pluripotent colonies to differentiate along a specific lineage. In particular, human iPSC within various *in vitro* contexts have been differentiated into endothelium[13-19]. Despite these reports, which describe the emergence of cell populations largely bearing molecular markers of the desired cell type, there is a paucity of functional characterization of the differentiated cell types. There are still significant unanswered questions about the character of the iPSC derived vascular cells, namely, whether they faithfully represent the mature phenotype and can perform the many dynamic functions of adult vascular cell types offering a surrogate model system for human primary cells. This functionality must be carefully assessed before their scientific and therapeutic potential can be realized[20].

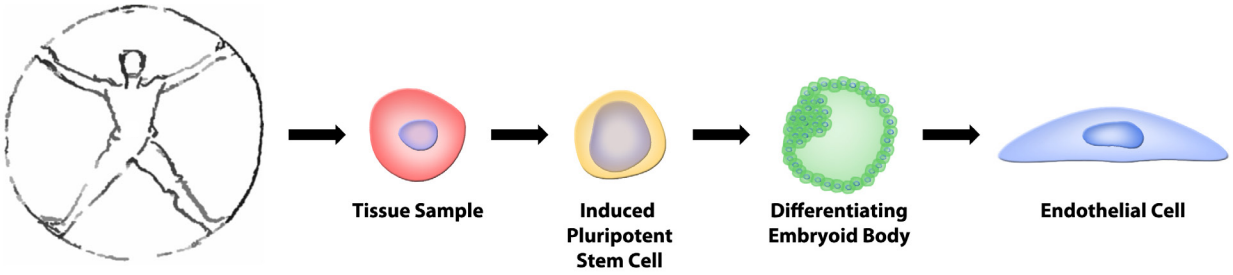

Figure 1.1 | Illustration of the process of generating endothelial cells from patient tissue samples via induced pluripotent stem cells.

At the outset of the study presented here, there existed only limited characterization of endothelia-specific functions within iPSC derived endothelium. Several groups have used human iPSC to generate endothelial cells and multiple reports describe the presence of endothelial protein markers by flow cytometry or immunofluorescence imaging, uptake of acetylated-LDL or the capacity to form networks when cultured on matrigel *in vitro* with iPSC endothelial cells[13-16,18,19,21]. One of these groups has demonstrated that subcutaneous implantation of a matrigel plug seeded with iPSC endothelial cells into immunocompromised mice results in the formation of erythrocyte-containing vascular networks after

2 weeks[14]. Additionally, two reports described that injection of human iPSC derived endothelial cells into immunocompromised mice with hind limb ischaemia induced by femoral artery ligation resulted in ameliorated peripheral perfusion[15,17]. In these three studies, the exogenous cells were shown to associate with the host vasculature; however, no cellular function was measured in the injected human endothelial cells. In fact, in these reports the regenerative effect of enhanced peripheral tissue perfusion was principally ascribed not to incorporation of human endothelial cells into the mouse vascular bed but to a paracrine effect of stimulating the existing vasculature to proliferate.

Despite these initial reports, it was previously unexplored whether human iPSC derived vascular endothelial cells are capable of complex physiological functions such as participating in the inflammatory response, initiating thrombosis, maintaing a dynamic barrier and responding to biomechanical stimluation, functions critical for maintaining vacular homeostasis. Elucidation of whether iPSC derived endothelium can display endothelia-specific functions is essential for applying iPSC endothelium to disease modeling. Hence, two of the goals of this study were to first reproducibly generate human iPSC-derived vascular endothelial cells (iPSC-EC) and, second, to then assess whether they could acquire specific functional phenotypes displayed by primary cultures of human vascular endothelium.

1.2 | Specification of vascular endothelium to arterial and venous fates

While all blood vessels are lined with a single cell layer of vascular endothelial cells, there exists significant heterogeneity amongst these endothelial cells depending on their anatomical location[22-27]. Hierarchy amongst blood vessels and anatomical differences between arteries and veins were first described by the fourth century BC physician Herophilos[28]. The modern conception of the circulatory system including the idea of a closed connect circuit with the contemporary directionality of blood flow would be described two thousand years later by William Harvey in his "De motu cordiis"[29]. The two most eminent categorizations of vascular endothelium in blood vessels are arterial and venous subtypes; however there are also microvascular endothelial cells as well as organ-specialized endothelium such as the blood brain barrier, kidney glomerular endothelium and liver sinusoidal endothelium. While there are additional hierarchies and dimensions of heterogeneity depending on the diameter of the blood vessel and also the local hemodynamic environment established by blood flow and the local vessel geometry, the variations between arterial and venous subtypes have been best characterized at the molecular level[30-34]. The ability of human iPSC derived vascular endothelium to acquire arterial and venous fates has been largely unexplored. Only during the writing of this dissertation was the first report published describing the presence of arterial and venous specific markers within a mixed heterogeneous pool of iPSC derived endothelium, though without isolating and purifying specific arterial or venous endothelial cells[35].

In order to generate vascular endothelial cells from human iPSC and further to specify them into arterial and venous fates, it is important to consider this synthetic *in vitro* differentiation process in relation to physiological human vascular development. In particular, one element of physiological development strikingly absent from *in vitro* differentiation is the presence of cardiac driven circulation and hence hemodynamics derived physical forces. It has long been known that biomechanical forces influence the vascular system during embryonic development. Such influence centered around two archetypical interactions: first, hemodynamic forces can alter the geometry of vascular networks, and second, hemodynamic forces can modify the arterial or venous identity of vascular endothelial cells.

There are numerous examples of how circulatory blood flow influences vessel geometry. For instance, within zebrafish expressing the silent heart mutation (in a gene encoding a cardiac myocyte troponin) which precludes cardiac contraction, the gross architecture of their arteries and veins develops; however, the lack of hemodynamic forces during development causes morphological and connectivity changes in secondary and tertiary vessel patterning[36]. Similarly, remodeling of the mouse yolk sac vasculature fails to develop proper vessel hierarchy from an initial plexus in embryos lacking circulation in both myosin light chain 2c knockouts[37] and sodium-calcium exchanger 1 knockouts[38].

In addition to influencing blood vessel network morphology, hemodynamic forces have been shown to affect the identity of vascular endothelium at the molecular level. In fact, multiple studies have described how the arterial or venous identity of vascular endothelial cells is able to be modified by hemodynamic forces both during embryonic development and during adulthood. For instance, quail arterial endothelial cells are able to integrate into chicken embryonic venous vasculature and express venous molecular markers while quail venous endothelial cells incorporate into arterial vessels and express arterial specific markers as a result of local hemodynamic forces[39]. Further, manipulation of the chick embryo vitelline vasculature by incision or ligation altering blood flow can influence the identity of the yolk sac vessels into arterial or venous following induction of arterial or venous flow respectively[40]. Transplantation of venous segments into the arterial tree of developing chick embryos induces the expression of ephrin-B2, a faithful marker of arterial endothelial identity[41]. This plasticity of vascular endothelial identity is not limited to the embryonic vasculature. It is commonly observed that when transplanting human venous segments into the arterial tree as commonly performed in coronary or peripheral artery bypass grafting, or in the gross alteration of blood flow such as in the artificial creation of arterial-venous fistulas, that the venous endothelium now exposed to an arterial hemodynamic microenvironment remodels and arterializes[42]. Several aspects of this arterialization can be replicated *in vitro* by exposing cultured primary human venous endothelial cells to arterial hemodynamic forces resulting in upregulation of arterial specific endothelial gene expression.

Although multiple observations demonstrated that there exists plasticity within the vascular endothelial arterial or venous identity subject to hemodynamic environment, new evidence arose describing the genetic specification of vascular endothelial fate. In 1998, Wang *et al* reported that embryonic murine vasculature selectively expresses the transmembrane ligand ephrinB2 on arterial endothelial cells and its cognate receptor EphB4 on venous endothelial cells prior to the onset of cardiac contraction, blood circulation and hence hemodynamic forces on the vascular wall[43]. This impressive observation demonstrated that arterial and venous endothelial cells initially exhibit molecular distinction through genetic developmental programs and not as a consequence of their biomechanical environment[34]. Subsequently, it was demonstrated that this early embryonic molecular distinction persists through development and post natal life[44]. Further, it has been reported that arterial and venous endothelial cells forming the first artery and vein in zebrafish arise from distinct angiogenic precursors[45].

As the ability to produce endothelium with arterial or venous identity from human iPSC has numerous applications for disease modeling and pharmacological testing, as will be explored in Chapter 6, the third goal of this dissertation is to isolate and characterize these specific endothelial subtypes. Specifically, as motivated by these previously published observations in the literature regarding endothelial specification, I sought to evaluate whether arterial and venous endothelium develops via genetic programs independent of hemodynamic condition and also whether distinct hemodynamic stimuli are able to affect endothelial specification.

1.3 | Specific aims of this dissertation

Specific Aim 1 | Develop a robust and reproducible methodology to generate vascular endothelial cells from human induced pluripotent stem cells

Specific Aim 2 | Evaluate endothelia-specific functionality in induced pluripotent stem cell derived endothelial cells

Specific Aim 3 | Examine the ability of human induced pluripotent stem cell derived endothelium to adopt arterial and venous specific identities

2 | The derivation of vascular endothelium from human induced pluripotent stem cells

2.1 | Differentiation of human induced pluripotent stem cells into vascular endothelium

In order to establish iPSC as a reliable source of vascular endothelium, several fundamental points needed to be addressed. While human iPSC have been shown to produce endothelial cells, the kinetics, purity, fidelity and reproducibility of endothelial differentiation within the spontaneously differentiating cell populations were not characterized. The following experiments sought to address these points, which are prerequisite for further use of iPSC endothelium as a practical experimental platform.

Human iPSC lines are able to be maintained in culture where they retain their undifferentiated pluripotent state and indefinitely self-renew under particular conditions as previously reported (see section 5.1). **Figure 2.1** presents a phase contrast image of a single colony of hundreds of iPSC (BJ1 iPSC line) growing on a feeder layer of mouse embryonic fibroblasts in their pluripotent state, in the presence of defined serum and high concentrations of bFGF. To induce differentiation and produce vascular endothelial cells, I differentiated human iPSC as embryoid bodies in suspension by physically lifting iPSC colonies from their substrate with a cell scraper and replacing the iPSC medium containing bFGF with differentiation medium containing 20% fetal calf serum. Complete details of the constituents of the media are given in Section 5.1. The specific serum chosen was selected from a screen optimizing proliferation and morphology of cultured human EC (previously performed by Ms. Kay Case, Brigham and Women's Hospital). During the course of my studies, I have used fetal calf serum produced from three different lots from the same vendor. There were no obvious inter-lot variations in the endothelial differentiation of iPSC. These differentiation culture conditions induced the iPSC to exit their pluripotent phase and begin differentiation into multiple cell types. As time passes and the embryoid bodies differentiate, they grow in large cystic spheroidal formats which can be observed by phase contrast microscopy as in **Figure 2.2** or alternatively seen by the unassisted eye in the culture dishes. This process

produces a heterogeneous population of presumably multiple cell types. To assess the time scale of when the differentiating embryoid bodies exit their pluripotent states and whether endothelial cell differentiation occurs, I performed quantitative real-time Taqman PCR (RT-PCR) using RNA harvested daily from whole embryoid bodies from the time of their generation from iPSC colonies (day 0) through 18 days of differentiation. Under these conditions, I observed a loss of expression of the transcription factors *Oct4* and *Nanog*, known to be critical for maintaining pluripotency[46], expression of *brachyury* suggesting differentiation through mesodermal germ layer lineage[47], expression of the developing hemangioblast transcription factor *Gata2*[48] and finally increasing expression of endothelial cell markers *VE-cadherin* (*VEC*), *KDR* (*VEGFR2*) and *CD31* (also known as *PECAM1*) (**Figure 2.3**). This gene expression analysis suggested that the embryoid bodies lost the transcriptional controls of pluripotency within a week and that endothelial cell development is occurring within approximately 7-14 day time frame. However, such analysis offers a crude perspective as the expression levels of such genes are averaged across several different cell types. There are several potential sources of heterogeneity in this differentiation process. There could be inter-embryoid body variation in the magnitude of endothelial cell differentiation. It is also possible that not all embryoid bodies are synchronized with respect to their developmental time. While the initial size of the embryoid body may be an important influence on its differentiation potential, it is difficult to precisely control the geometry during embryoid body formation. It is also likely that with small numbers of cells there exist stochastic processes dictating cell differentiation decisions into the three germ layers and more mature cell types which may be influence by local cues difficult to control. To better define the presence and quantity of the putative endothelial cell population which is suggested by the qRT-PCR results, I measured the cell surface protein expression of endothelial cell markers VEC, CD31 and KDR with flow cytometry within dissociated embryoid bodies after different durations of differentiation. As seen in **Figure 2.4**, after 10 days of differentiation, a VEC+/CD31+ endothelial cell population peaked at 18% ± 4% (mean ± s.d.) as the KDR+/CD31+ population was 14.7% ± 4% (mean ± s.d.) of the embryoid body. To visualize the presence of endothelial cells within the intact embryoid body with approximately 1 mm diameter on day 10, I used two photon

confocal microscopy with immunofluorescent-labeled VEC which illustrated that most VEC+ cells reside in network structures (**Figure 2.5**). While this differentiation process from iPSC into vascular endothelial cell was reproducible within the iPSC line tested (BJ1 cell line), I investigated whether such *in vitro* endothelial differentiation is robust and generalizable to other human iPSC lines. To confirm this, I evaluated the endothelial differentiation potential of several other iPSC lines derived from different laboratories, from different donor cell types and generated through different induced pluripotency reprogramming technologies (see section 5.1 for details regarding each line). Each of the 4 lines tested was reproducibly capable of producing endothelial cells as measured by the presence of VEC expressing cells by flow cytometry after 10 days of differentiation; however, the fraction of VEC+ cells differed between the lines (**Figure 2.6**). Due to its high yield of endothelial cells, the cell line BJ1 generated from human foreskin dermal fibroblasts by the overexpression of *Oct4*, *Sox2*, *KLF4* and *c-myc* (the so called Yamanaka factors) by four retroviruses was utilized for analysis in subsequent experiments described in the following sections.

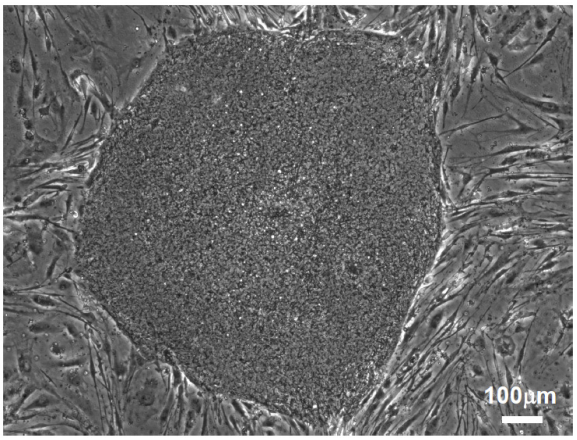

Figure 2.1 | Phase contrast image of a colony of human induced pluripotent stem cells in their pluripotent state cultured on a feeder layer of mouse embryonic fibroblasts.

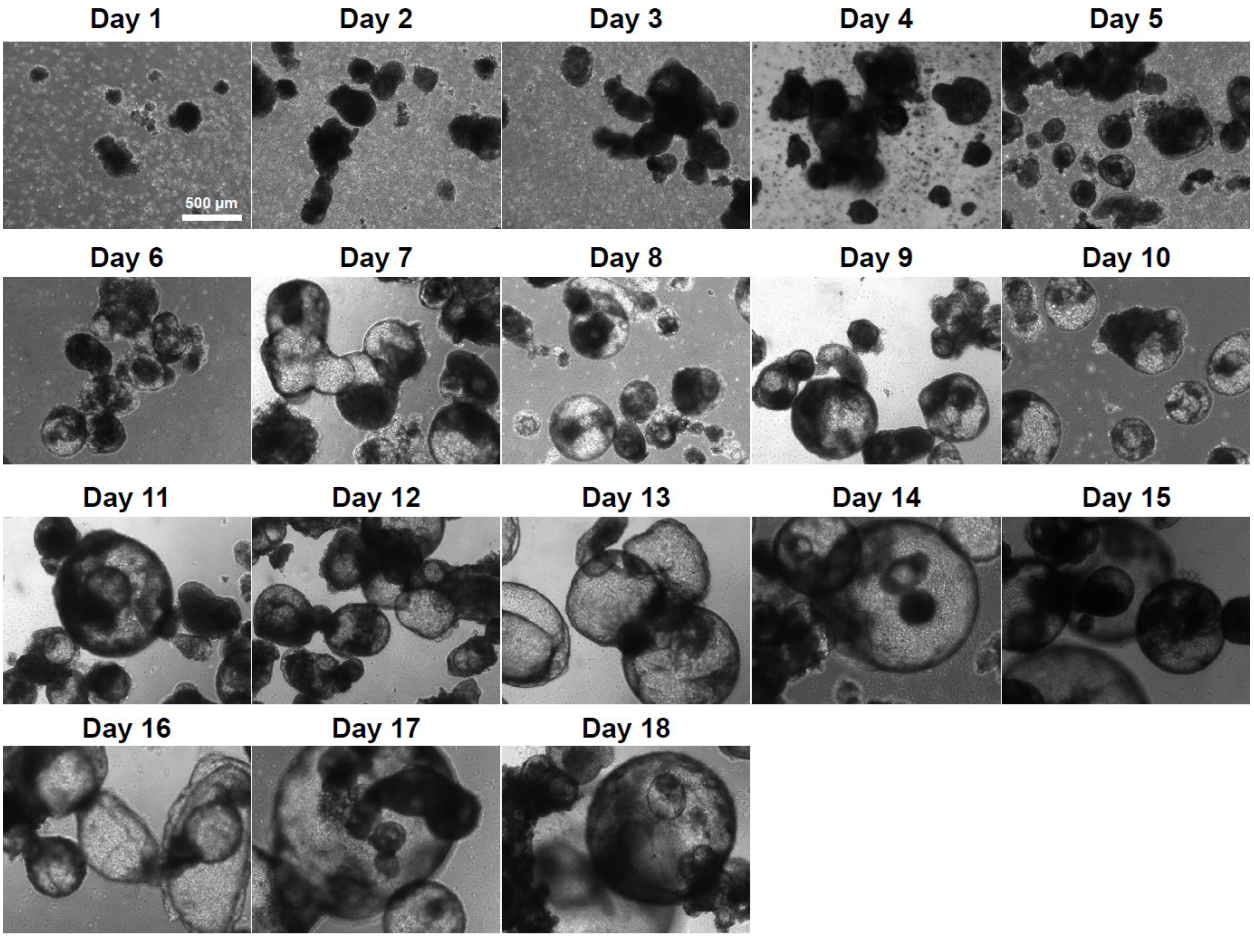

Figure 2.2 | Phase contrast images showing temporal profile of spontaneously differentiating iPSC as embryoid bodies acquired after various durations of differentiation.

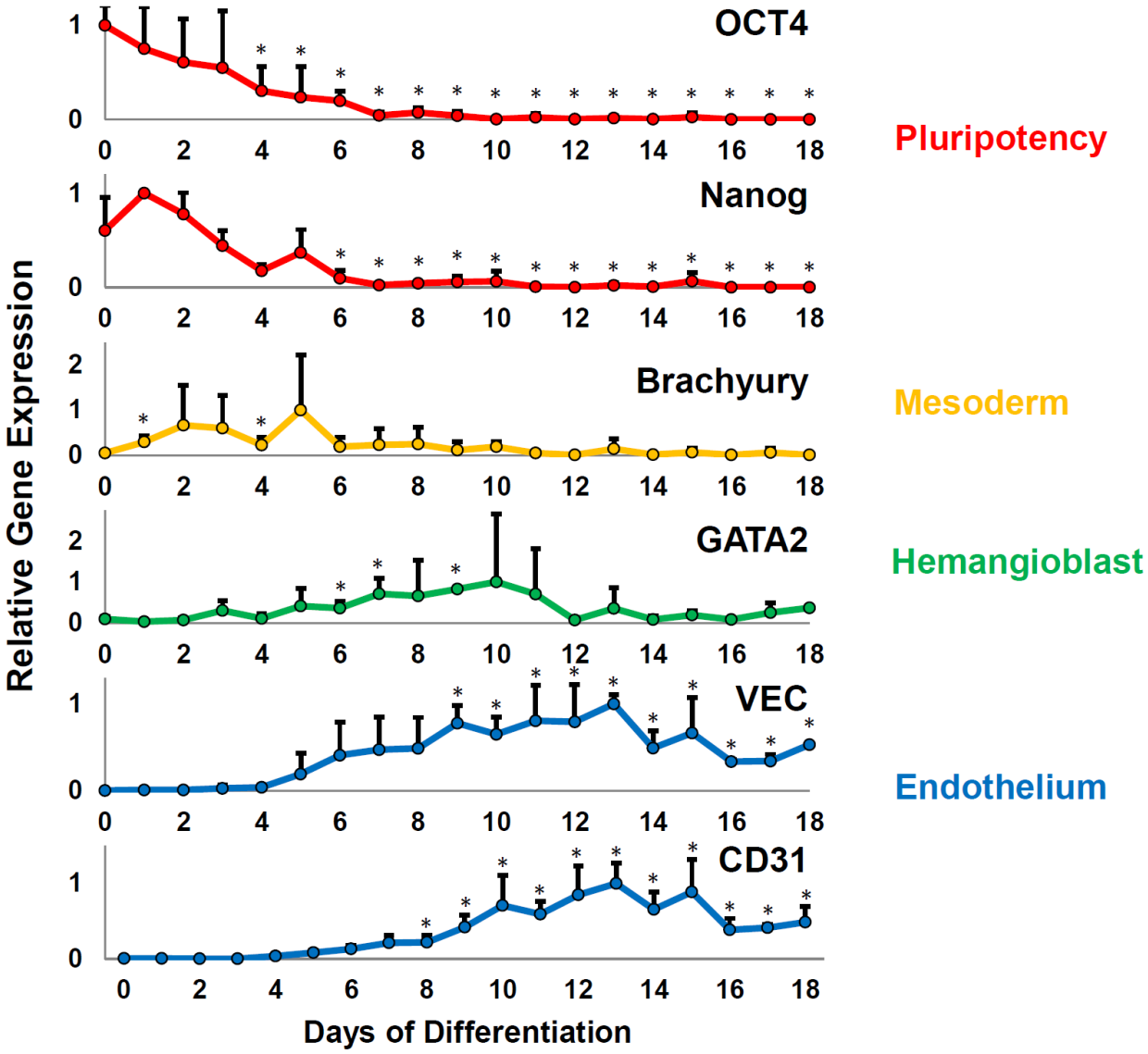

Figure 2.3 | Temporal profiles of gene expression of indicated genes in spontaneously differentiating embryoid bodies after different durations of differentiation as measured by qRT-PCR. Error bars indicate standard errors, n=3. * indicates $p < 0.05$ comparing indicated mean to the baseline undifferentiated day 0 mean.

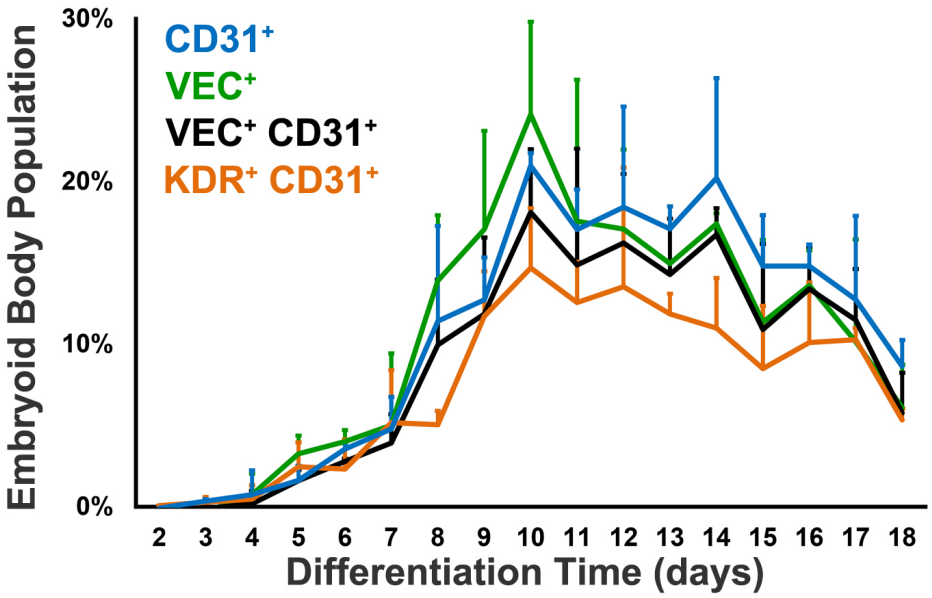

Figure 2.4 | Temporal profile of the fraction of embryoid body cells comprised of CD31+, VEC+, VEC+/CD31+, KDR+/CD31+ cell populations as measured by flow cytometry. Error bars indicate standard errors, n=3.

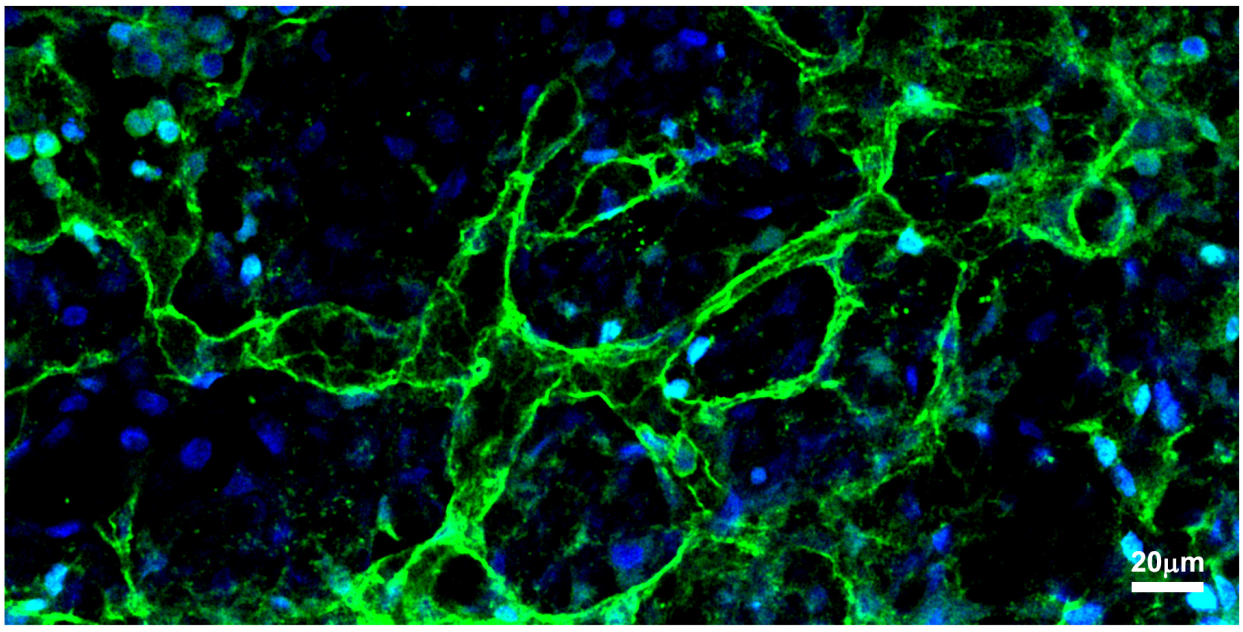

Figure 2.5 | Reconstruction of three dimensional two-photon confocal microscopy images within an intact day 10 embryoid body with immunofluorescent labeled VE-cadherin (green) and nuclei (blue). VE-cadherin expressing endothelial cells grow in vessel-like networks in the developing embryoid body.

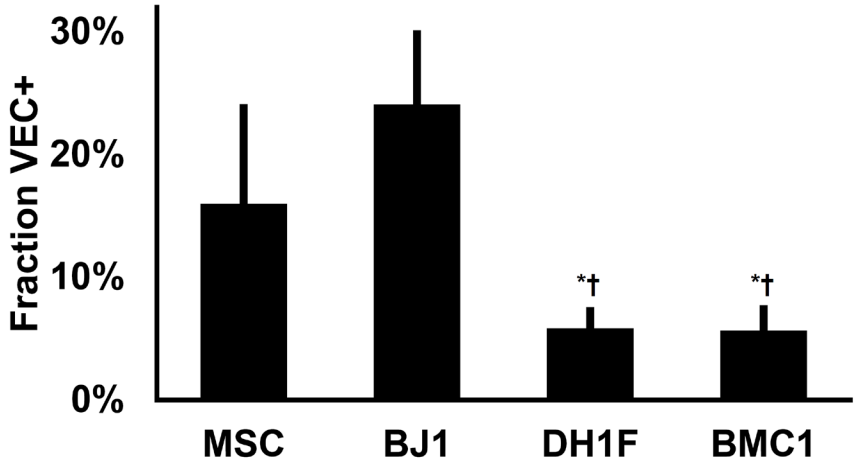

Figure 2.6 | Four different iPSC lines were used to generate embryoid bodies. The fraction of day 10 embryoid bodies expressing VE-cadherin was measured by flow cytometry. Error bars are standard deviation, n=3-4. * indicates a statistically different mean from MSC with $p < 0.01$ and † indicates a statistically different mean from BJ1 with $p < 0.01$.

2.2 | Isolation of the vascular endothelial population

After understanding the reproducibility and dynamics of the temporal profile of endothelial differentiation from the embryoid bodies, the next step I undertook was to develop a protocol to isolate a purified endothelial cell population from the heterogeneous embryoid bodies. After this isolation, I subcultured this endothelial population *in vitro* for further characterization and downstream applications. The following experiments describe the optimization process for isolating and subculturing vascular endothelial cells from the embryoid body for further analysis.

I choose to isolate endothelial cells from the embryoid body on day 10 of differentiation as the VEC+/CD31+ population peaks at day 10, providing sufficient yield of endothelial cells, high purity of endothelial cells in the presorted population and practicality of limiting the duration of experiments. Previous studies have differentiated human pluripotent stem cells and isolated endothelial cells based on cell surface expression of CD31, CD34, or KDR antigens[13-19]. In contrast, I choose to isolate endothelial cells from the embryoid body based on VEC expression, the most definitive marker of endothelial identity, as I observed the presence of putatively non-endothelial CD31+, and KDR+ cells that did not express VEC within the embryoid body (**Figure 2.7**, left and center panel). While isolation based upon multiple markers is desirable, I observed poor survival based on plating efficiency after fluorescence assisted cell sorting while magnetic bead based separation improved viability. Dissociated embryoid bodies were exposed to mouse IgG1 anti-VEC phycoerythrin-conjugated antibody then washed and exposed to magnetic bead conjugated anti- phycoerythrin antibody. Following this, the cells were passed through a positive selection magnetic bead assisted cell sorting (MACS) column according to manufacturer's instructions (Miltenyi). Magnetic bead sorting produced a population with 95% ± 3.7% (mean ± s.d., n=4) VEC+ cells (**Figure 2.7**, right panel).

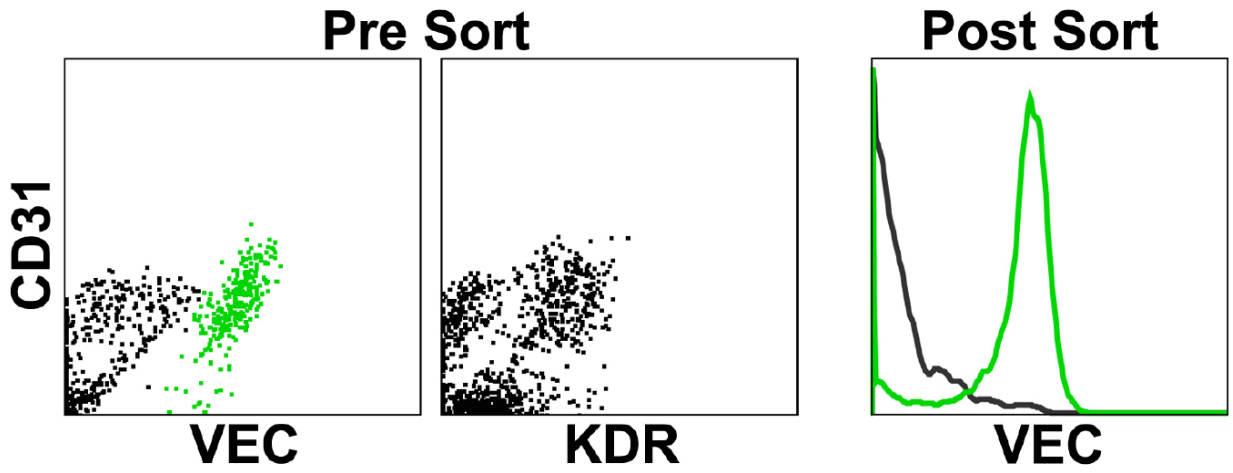

Figure 2.7 | Endothelial population labeled with CD31, VEC and KDR visualized by flow cytometry before sorting (left) and labeled with VEC after magnetic bead sorting (right).

Before endothelial cell isolation, the large embryoid bodies must be dissociated into single cells for labeling and sorting. The development of a protocol to isolate iPSC derived endothelial cells began based on a previously published method for the dissociation of human embryonic stem cell derived embryoid bodies for hematopoietic cell harvesting[49]. This protocol called for the enzymatic dissociation of the embryoid bodies yet following this protocol resulted in incomplete digestion of the iPSC embryoid bodies after the specified two hours of enzymatic treatment. To optimize this methodology for iPSC derived embryoid bodies and for endothelial cell harvesting, I screened a panel of enzymes for their ability to efficiently dissociate the embryoid bodies into single cells. The embryoid bodies were washed with PBS+ then treated for 2 hours at 37°C agitated in a table top shaker at 1100 rpm with one of several different enzyme treatments: collagenase-dispase (Roche), Cell Dissociation Buffer (Invitrogen), Liberase (five different variants: TL, TM, DL, DH, TH, all from Roche), dispase (Roche), collagenase IV (Invitrogen), collagenase B (Roche) and dispase-DNAse (Roche and Invitrogen). After enzymatic treatment, the slurry was washed with PBS- then treated with enzyme-free Cell Dissociation Buffer (Invitrogen) for 20 min at 37°C shaking at 1100 rpm. After this step, I triturated the slurry with a P1000 pipette tip and passed it through a 70μm then 40μm mesh filters. To select the most efficient enzyme, I

analyzed the filtered slurries by flow cytometry. I created a Dissociation Index defined as the product of the fraction of flow cytometry events contained within each of the three gates in **Figure 2.8A** to be able to quantify the effects of the enzyme on dissociating the embryoid body into single viable endothelial cells. Specifically, these gates select events of the size of cells on forward and side scatter dimensions that are alive and exclude propidium iodide, and express VEC. The product of these fractions should estimate the yield of VEC+ cells following enzymatic dissociation for a given starting population. The value of this Dissociation Index for the enzyme panel is shown in **Figure 2.8B**. I selected the four enzymes with the highest Dissociation Index values, repeated the embryonic body dissociation then followed by sorting the filtered slurry for VEC+ cells with magnetic bead sorting to ensure that the endothelial fraction remains viable after being passed through the sorting column (**Figure 2.8C**). This optimization process identified 2 mg/mL collagenase B as the best candidate for enzymatic digestion of the embryoid bodies; hence, it was used for the remainder of the following experiments. In general, independent experiments were defined as independent differentiations spanning 10 days and isolations beginning from a different batch of iPSC.

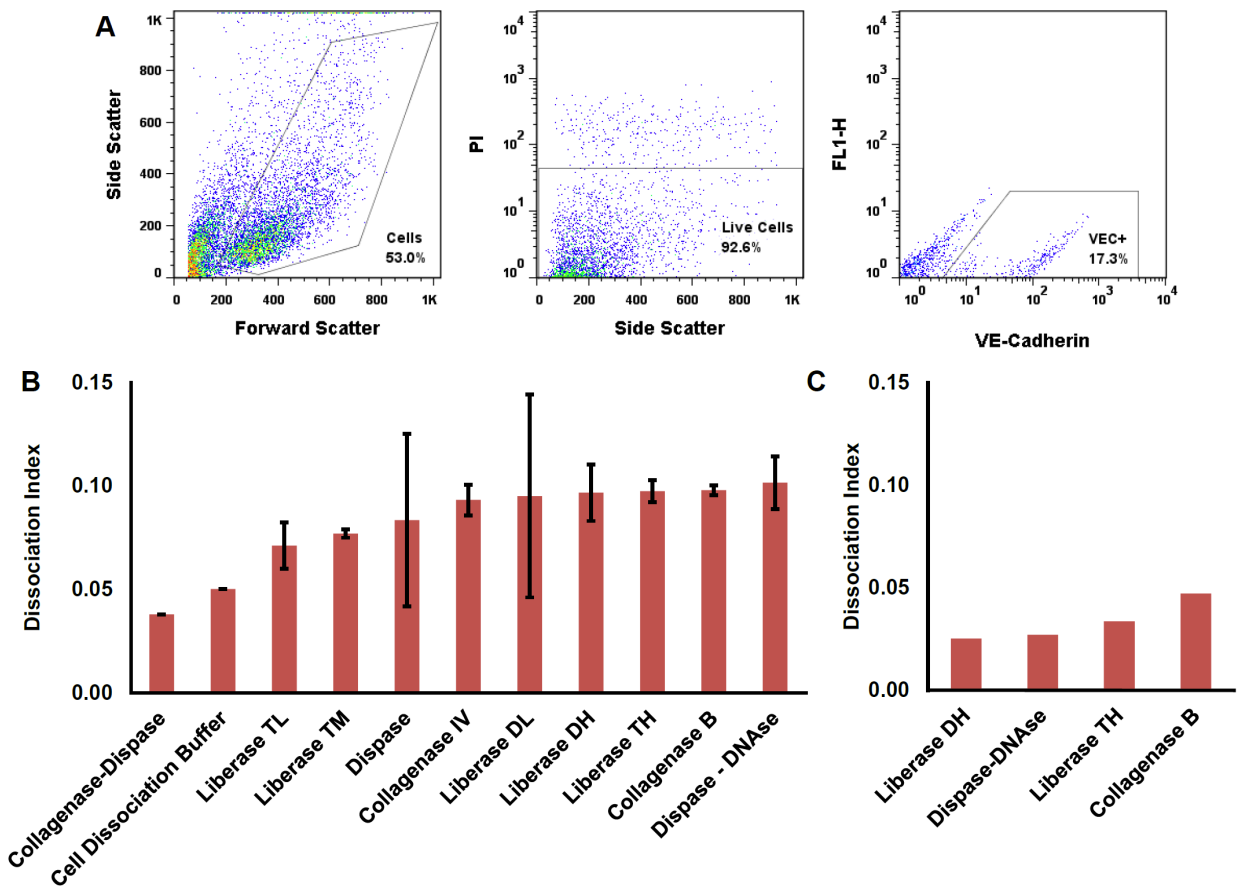

Figure 2.8 | (A) Gating of dissociated embryoid bodies showing the three flow cytometry gates comprising the Dissociation Index. (B) The value of the Dissociation Index for a panel of enzymes after dissociating embryoid bodies before sorting. Error bars indicate standard deviations, n=3. (C) Dissociation Index values in the VEC+ magnetic bead sorted population for selected enzymes.

After dissociation and magnetic bead based isolation, the purified VEC+ endothelial cell population was plated into *in vitro* culture. Initially, I attempted to culture the iPSC derived vascular endothelial cells on gelatinized (0.1% gelatin) tissue culture plastic surfaces following the protocol typically used for human umbilical vein endothelial cell culture in our laboratory; however, I noticed poor cell spreading immediately after plating and large numbers of cells delaminating within the first 24 hr after seeding. This observation suggested that the *in vitro* culture conditions, perhaps the extracellular matrix of 0.1% gelatin, was not be ideal for iPSC derived endothelial cell growth. To evaluate other extracellular matrices, I isolated VEC+ cells and cultured them using a Millicoat ECM Screening Kit (Millipore, Billerica, MA) which provides a standard multiwell plate with surface pre-coated surfaces with several different cell matrix proteins. Out of the matrices provided (fibronectin, collagen I, collagen II, collagen IV, laminin, vitronectin, tenascin and bovine serum albumin), only the fibronectin coated well exhibited any adhesion and spreading in cells 4 hr after plating (**Figure 2.9**). Following this initial observation, I sorted the VEC+ cells onto tissue culture plastic surfaces that I plasma treated to enhance protein adsorption and coated with 50 μg/mL of human serum fibronectin with volume equivalent of 2.5 μg fibronectin / cell culture area cm^2 overnight at 37°C which vastly improved spreading compared to the commercial kit. Following this procedure, dissociating with collagenase B, using magnetic bead based sorting and plating onto self-made fibronectin surfaces at 20,000 cells/cm^2, produced a robust protocol for the isolation of VEC+ endothelial cells from iPSC. **Figure 2.10** presents a phase contrast image of one such isolation after 3 days of culture. This procedure was used to isolated endothelial cells from embryoid bodies for all the following experiments.

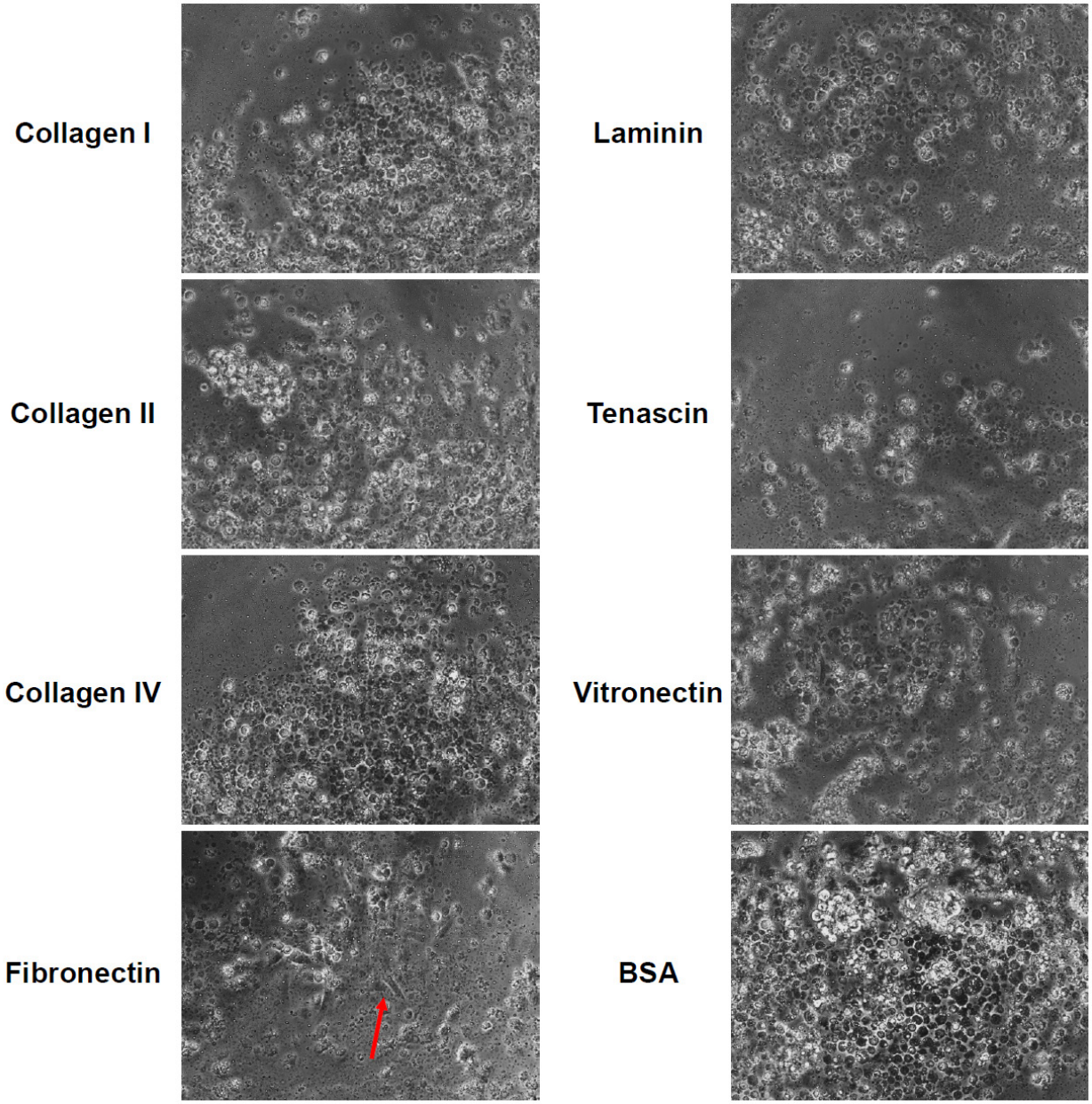

Figure 2.9 | Evaluation of iPSC endothelial cell spreading on various matrices 4 hours after magnetic bead sorting of dissociated embryoid bodies for VEC+ cells and plating.

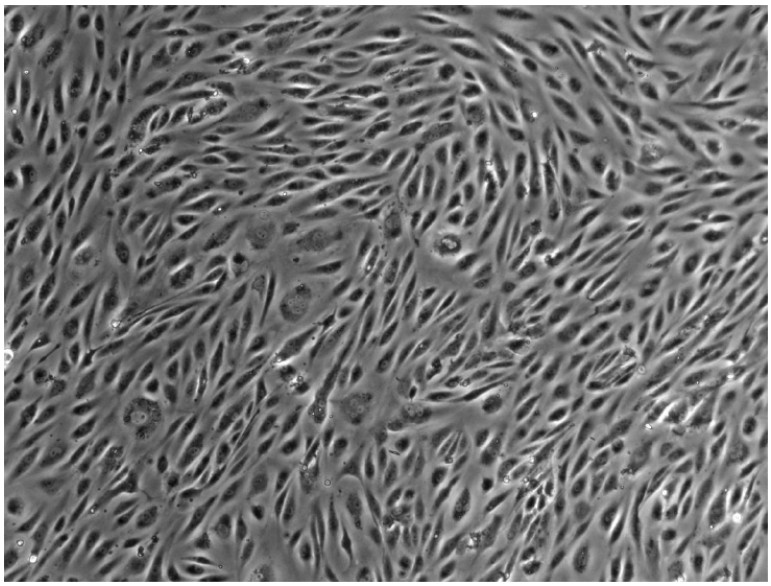

Figure 2.10 | VEC+ endothelial cells isolated from embryoid bodies, purified with magnetic bead sorting and cultured on fibronectin for 3 days.

3 | The functional phenotypes of human induced pluripotent stem cell derived vascular endothelium

3.1 | Characterizing endothelial identity by molecular characteristics and behavior

After I developed and validated a robust protocol to generate purified VEC+ cells derived from human iPSC, I sought to evaluate whether these VEC+ cells bear basic molecular and functional fidelity to primary human endothelium which is a precondition for their use in patient specific cell based models of endothelial function. Hence, I assessed whether isolated iPSC endothelial cells exhibit the expression of characteristic endothelial markers and behavior after *in vitro* culture following isolation from embryoid bodies. Endothelial cells were plated and cultured for 3 days (passage 1) after which characterization was performed. As seen in **Figure 3.1**, iPSC endothelial cells displayed typical cobble-stone morphology, expressed VEC and CD31 at cell junctions and endothelial nitric oxide synthase (eNOS) at the perinuclear region and plasma membrane as seen by immunofluorescent imaging. This observed subcellular localization of eNOS is similar to the compartmentalization of eNOS in intact human vessel endothelium where eNOS is known to localized to the Golgi complex and with plasmalemmal caveolin-1[50,51]. Further, the cell surface expression of VEC, KDR and the anti-thrombotic thrombomodulin on the iPSC endothelial cells is nearly uniform as measured by flow cytometry plotted in **Figure 3.2**.

The endothelial glycocalyx is a large ~0.5 μm thick complex heterogeneous extracellular organelle between the endothelial plasma membrane and the vessel lumen. This structure consists of a network of glycoproteins and associated integral carrier proteins[52,53]. The endothelial glycocalyx is believed to play a role in vascular permeability, transendothelial cellular transmigration and given its integration with the cytoskeleton and position at the interface between the moving blood in the vessel lumen and the stationary endothelial cell is hypothesized to be a mechanosensor. I investigated whether the iPSC derived endothelium expressed several protein constituents of the glycocalyx. I observed that the

glycoproteins heparan sulfate and chondroitin sulfate as well as the carrier proteins syndecans-1,2 and 4, glypican-1, and CD44 are expressed on the surface of the iPSC endothelial cells as measured by flow cytometry (**Figure 3.3**). It has been observed that the expression levels of glycocalyx proteins is influenced by shear stress *in vitro*[54,55] and correlates with shear stress levels *in vivo*[56,57]. It has also been observed that the there is a fibrous extracellular glycocalyx structure present in the embryonic quail dorsal aorta and heparan sulfate and chondroitin sulfate in the murine yolk sac at the same developmental time as the onset of circulation[58]. These observations suggest that the development of the glycocalyx is dependent on the biomechanical stimulation. My results with human iPSC derived endothelium suggest that blood flow is not necessary for the initial surface expression of the various protein constituents of the glycocalyx, though it may likely later influence their expression and assembly into the mature glycocalyx layer.

I also evaluated several behaviors of vascular endothelial cells typically observed within *in vitro* culture. Vascular endothelial cells are known to avidly uptake acetylated low density lipoprotein (AcLDL) particles via scavenger receptor mediated endocytosis[59,60]. I assessed whether iPSC-EC exhibit this capability by exposing the cells to 10 μg/mL of fluorescently labeled acetylated low density lipoprotein (DiL-AcLDL) in culture supernatant for 5 hrs. The iPSC endothelium endocytosed the DiL-AcLDL particles producing a punctate patterning observed by immunofluorescent imaging (**Figure 3.4B**) suggesting cytoplasmic vesicular internalization of the particles. Flow cytometry indicated that nearly the entire population of endothelial cells had internalized the labeled AcLDL (**Figure 3.4C**).

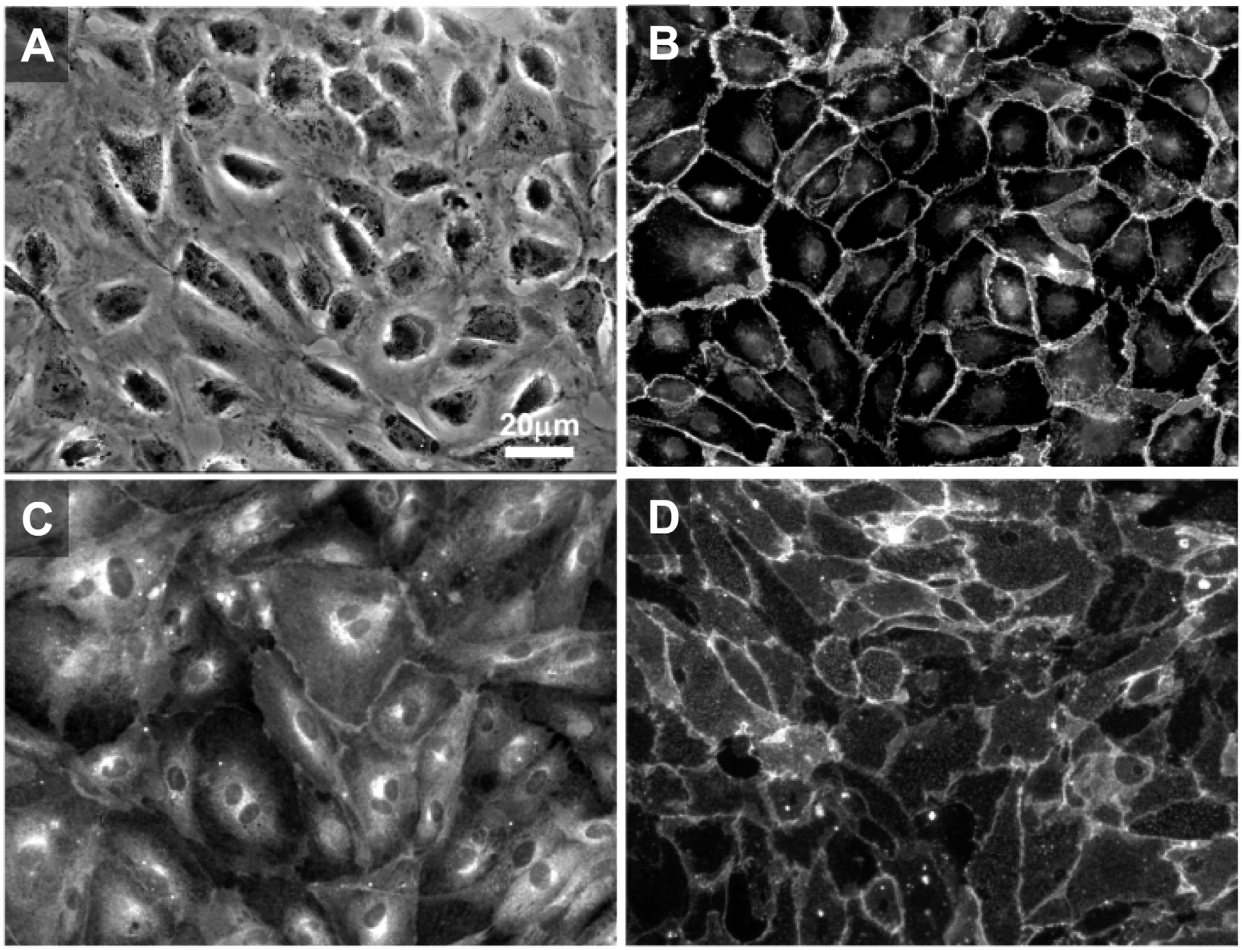

Figure 3.1 | (A) Phase contrast image of iPSC endothelium. Immunofluorescent stain of (B) VEC, (C) eNOS and (D) CD31

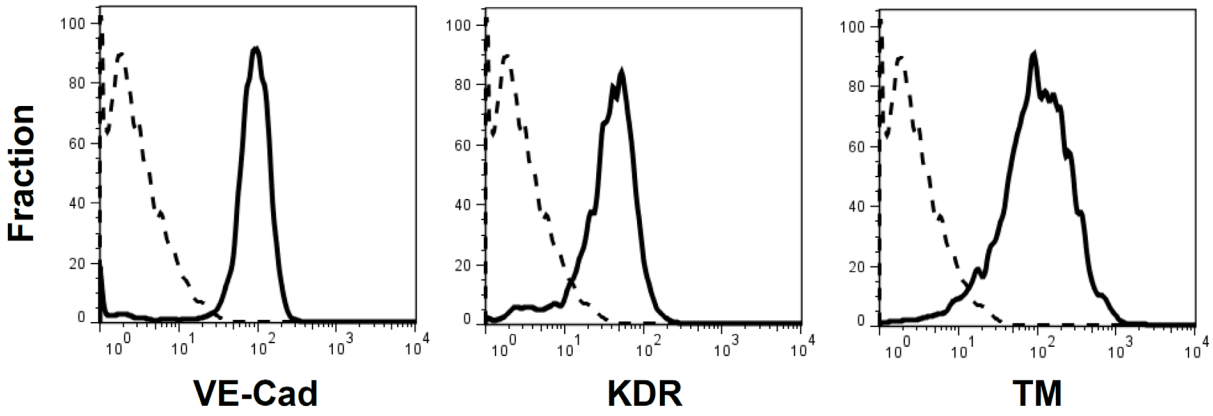

Figure 3.2 | Expression of VEC, KDR and thrombomodulin on the iPSC endothelial cell surface measured by flow cytometry.

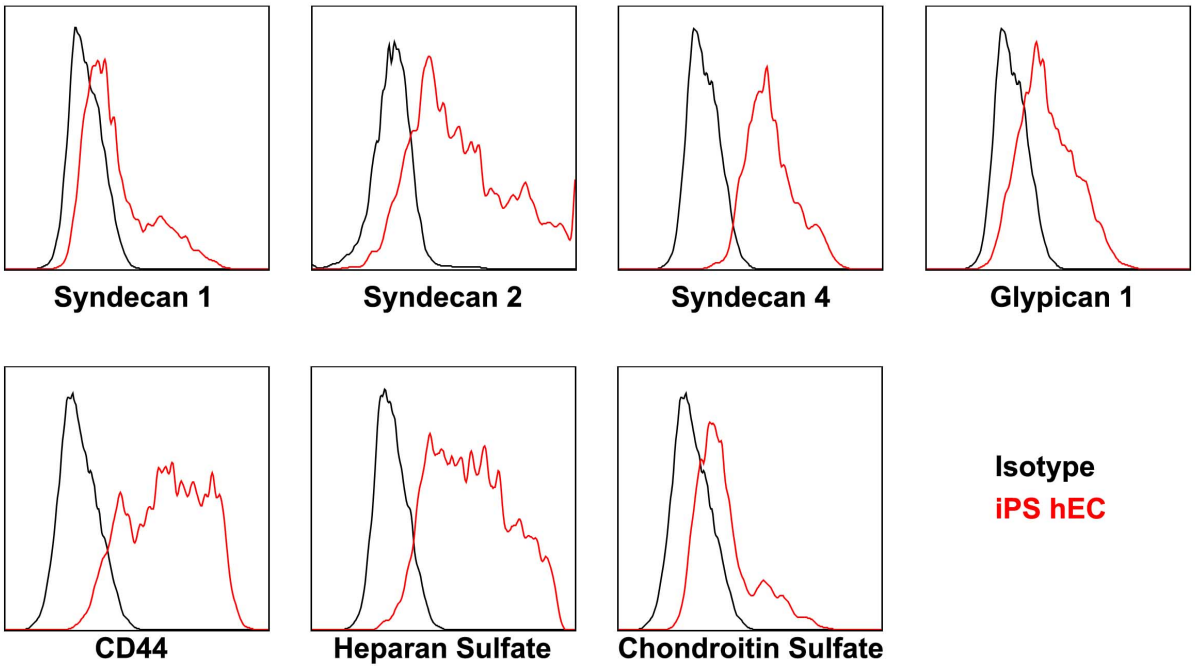

Figure 3.3 | Expression of extracellular glycoproteins and carrier proteins comprising the endothelial glycocalyx measured by flow cytometry.

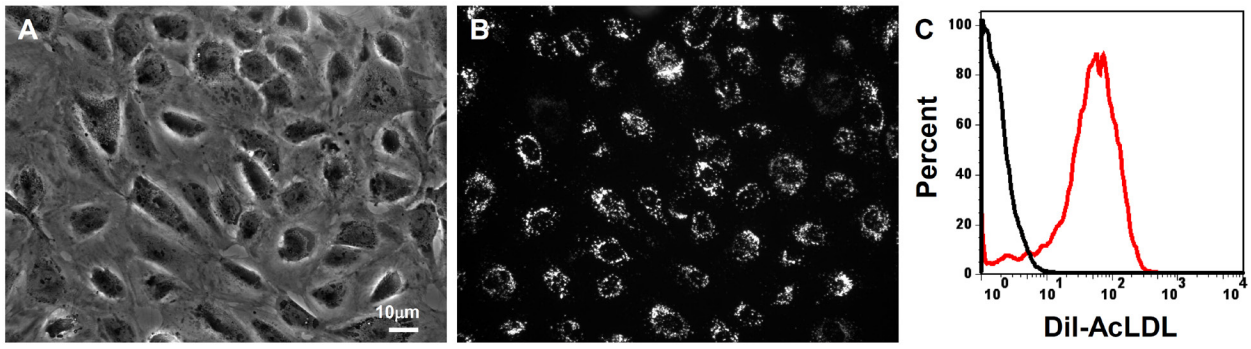

Figure 3.4 | (A) Phase contrast image of cultured iPSC endothelium. (B) Fluorescent image of internalized DiL-conjugated acetylated-LDL in punctate appearance. (C) Uniformity of acetylated-LDL uptake measured by flow cytometry; DiL-AcLDL untreated (black) and treated (red).

One of the fundamental functions of the vascular endothelium is to develop new blood vessels from existing ones. This process is controlled through local balance of pro- and anti-angiogenic factors, pathways and genes. There are several methodologies to assess angiogenic potential within cultured endothelial cells generally fitting into two categories: network formation on two dimensional extracellular matrix[61] or sprouting from a carrier through a three dimensional matrix[62]. As seen in **Figure 3.5**, when cultured on matrigel or matrigel when in the presence of 35 ng/mL bFGF or 50 ng/mL VEGF, the iPSC endothelial cells assembled into a network formation. This capability may be important for several of the putative applications of iPSC vascular endothelium both explored in section 6.2: angiogenic cell based therapy and genetic disease modeling.

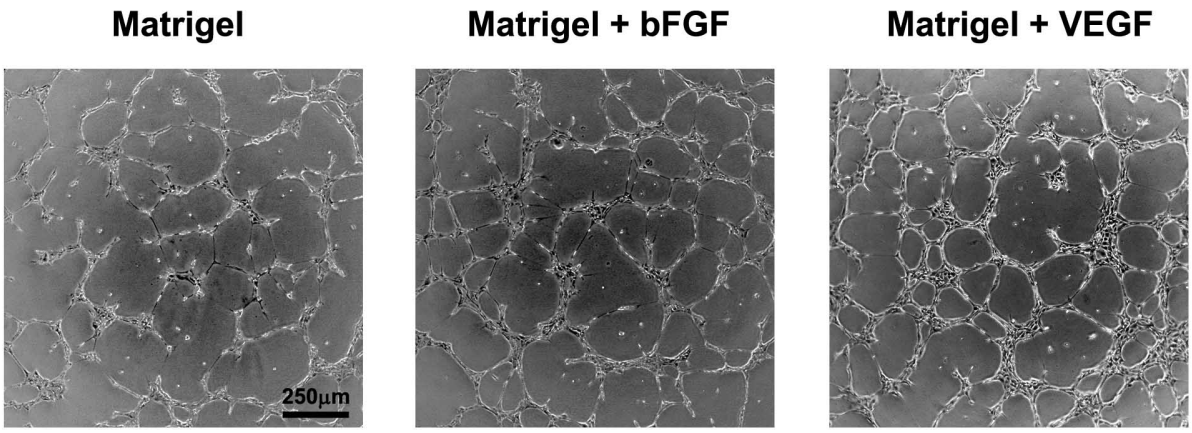

Figure 3.5 | Phase contrast images of networks formed after culture on low-serum matrigel in the presence of 35 ng/mL bFGF or 50 ng/mL VEGF after 6 hr.

3.2 | Characterization of functional Weibel-Palade Bodies

The initiation of thrombosis, the coagulation of blood, is a critical function of vascular endothelial cells. A principal component of this physiologic process is the attachment of platelets to the endothelium following vascular injury mediated by endothelial von Willebrand Factor (vWF). Endothelial cells synthesize vWF and store the large multimeric glycoprotein within endothelia-restricted intracellular storage granules termed Weibel-Palade Bodies[63]. Production of Weibel-Palade Bodies is an essential component of the hemostatic and thrombotic function of vascular endothelium and not previously explored in endothelial cells derived from human iPSC. In order to assess the presence of these organelles in iPSC-EC, I investigated with immunofluorescence imaging whether iPSC derived endothelium expresses vWF and another protein known to be stored in Weibel-Palade Bodies, angiopoietin-2[64]. These experiments revealed, as shown in **Figure 3.6A and B**, that both vWF and angiopoietin-2 are present in iPSC endothelium in a punctate pattern suggesting their localization in Weibel-Palade Bodies as in primary endothelium. To verify these observations, I examined the ultrastructure of iPSC endothelial cells by transmission electron microscopy and observed electron-dense striated structures with morphology characteristic of Weibel-Palade Bodies[65-67] as seen in micrograph in **Figure 3.6C**. I also confirmed that these Weibel-Palade Bodies identified by electron microscopy contain vWF by observing immunogold particle-labeled vWF localized to these structures as in **Figure 3.6D**. Functionally, Weibel-Palade Bodies store and rapidly exocytose their cargo including vWF and P-Selectin to initiate a hemostatic response[68,69]. Thus, in order to verify that the Weibel-Palade Bodies were competent for rapid exocytosis, I exposed the iPSC endothelial cells to histamine and measured cell surface expression of another Weibel-Palade Body constituent, P-Selectin via immunofluorescence. I observed rapid and transient presentation of P-Selectin to the cell surface (**Figure 3.6E**). These observations are particularly important for the potential of iPSC endothelium to be used in the human body as a cell based therapy and also for the potential application to modeling diseases, in particular the von Willebrand Disease class of hemophilias caused by mutations in the *vWF* gene.

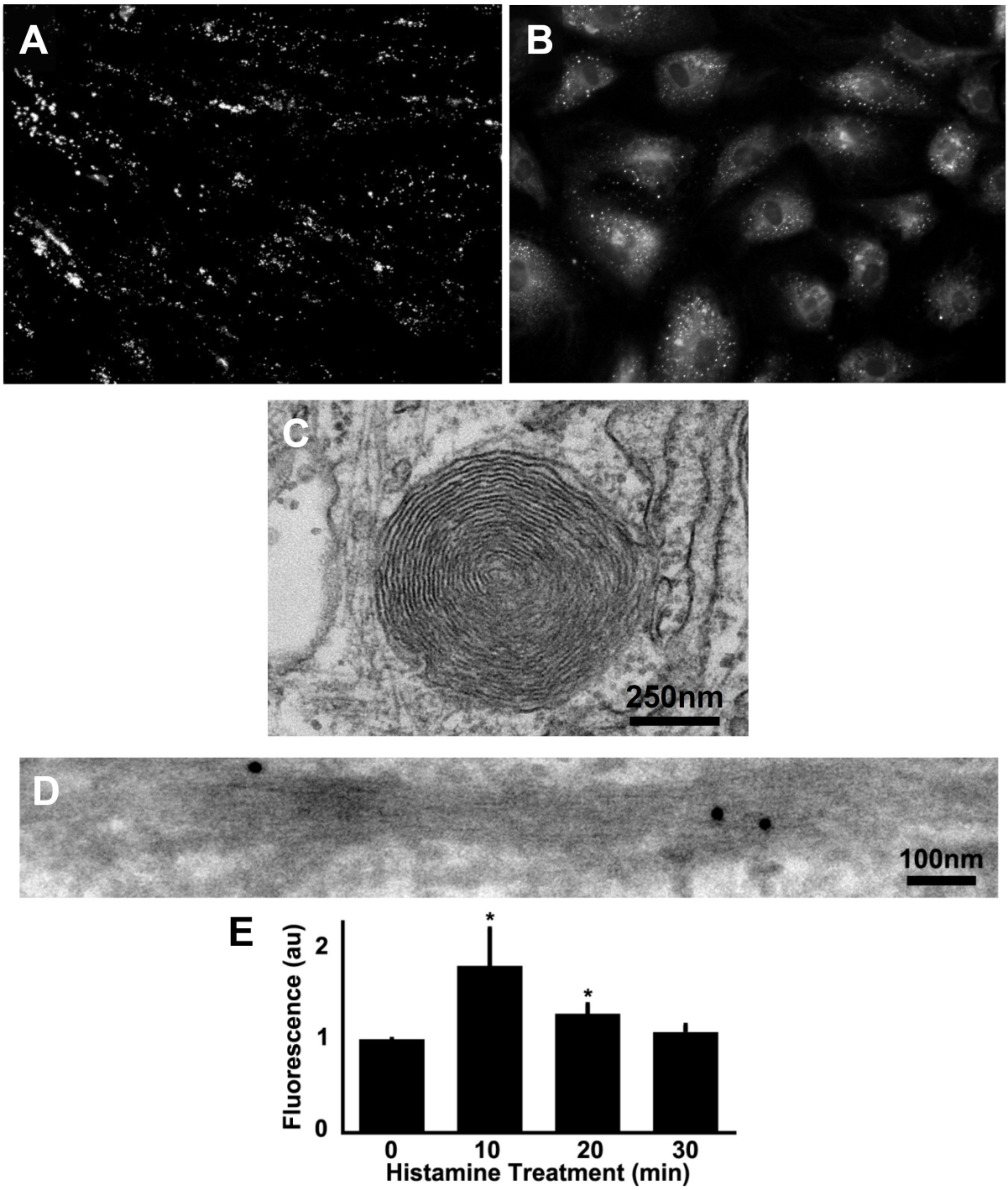

Figure 3.6 | (A) Immunofluorescent staining of (A) vWF and (B) angiopoietin-2. (C) Transmission electron micrograph of an electron-dense striated structure resembling a Weibel-Palade Body. (D) Electron micrograph with vWF-immunogold labeling of a Weibel-Palade Body. (E) Surface expression of P-Selectin after 10μm histamine treatment. Data are normalized to reading at 0 min and shown as mean ± s.d.. * indicated statistically different means compared to 0 min with $p < 0.05$, n=4.

3.3 | Assessment of endothelial activation

After observing several molecular and behavioral features of mature vascular endothelium within the iPSC derived endothelial cells above, I investigated whether iPSC derived vascular endothelium can display the multiple functional phenotypes characteristic of mature endothelium. A pathophysiologically essential property of the endothelium is to respond to pro-inflammatory stimuli and participate in inflammatory response. In response to pro-inflammatory stimuli, the endothelial cells can adopt an activated phenotype characterized by the secretion of pro-inflammatory cytokines and chemoattractants and presentation of leukocyte adhesion molecules at its surface[70,71,72,73]. This coordinated response can promote the interaction of monocytes, neutrophils, and T cells and the vascular wall mediating many inflammatory processes.

To assess whether iPSC derived endothelial cells are capable of exhibiting this functional phenotype, I exposed iPSC endothelial cells to several pro-inflammatory stimuli, namely interleukin-1 beta (IL1β), tumor necrosis factor alpha (TNFα) and lipopolysaccharide (LPS), and assessed the cell surface expression of the adhesion molecules E-Selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), all critical for endothelial-leukocyte interactions (**Figure 3.7A**). Treatment with IL1β, TNFα or LPS for 6 hr induced the expression of E-selectin and ICAM-1 in the majority of the cell population, and of VCAM-1 in a fraction of the population. After 24 hr of IL1β, TNFα or LPS stimulation, the iPSC endothelial cells expressed E-Selectin to a lesser degree, and ICAM-1 and VCAM-1. The temporal difference in expression of E-Selectin, ICAM1 and VCAM-1 are similar to those observed in primary human endothelial cells. Additionally, treatment with interferon-gamma (IFNγ) induced the iPSC endothelial cells to upregulate ICAM-1 expression synergistically with TNFα in a time-dependent manner (**Figure 3.7B**) as reported in studies of primary endothelial cell cultures[74].

In addition to presenting adhesion molecules, the activated endothelial phenotype is characterized by secretion of pro-inflammatory cytokines responsible for attracting immune cells. Therefore, I

examined whether iPSC endothelial cells have this capability by measuring the concentration of several pro-inflammatory cytokines in the culture supernatant in response to pro-inflammatory activation. Treatment for 24 hr with IL1β, TNFα or LPS induced the iPSC-EC to secrete monocyte chemotactic protein-1 (MCP1), interleukin-8 (IL8), RANTES and interferon-gamma-induced protein 10 (IP10), potent chemoattractants that act on T cells, neutrophils and monocytes, into the cell culture supernatant which I measured by cytometric bead assay (**Figure 3.8**). Additionally, treatment with interferon-gamma (IFNγ) induced the iPSC endothelial cells to secrete IP10 and monokine induced by gamma interferon (MIG).

Importantly, the coordinated expression of leukocyte adhesion molecules and secretion of pro-inflammatory cytokines by the vascular endothelium orchestrates the transendothelial migration of leukocytes. Given that the iPSC derived endothelial cells exhibited inducible leukocyte adhesion molecule surface expression and secretion of pro-inflammatory chemoattractants, I investigated whether these endothelial cells are also able to support interaction with leukocytes. After stimulating the iPSC endothelial cells with 4 hr treatment with TNFα, I observed that they were able to coordinate the integrated behavior of inducing human neutrophil and T lymphocyte rolling, arrest, and transmigration under laminar fluid flow (**Figure 3.9A and B**). The kinetics and fraction of transmigrating leukocytes were comparable to those seen with primary umbilical vein EC, the *in vitro* human model currently most commonly used for leukocyte transmigration studies (**Figure 3.9C-E**).

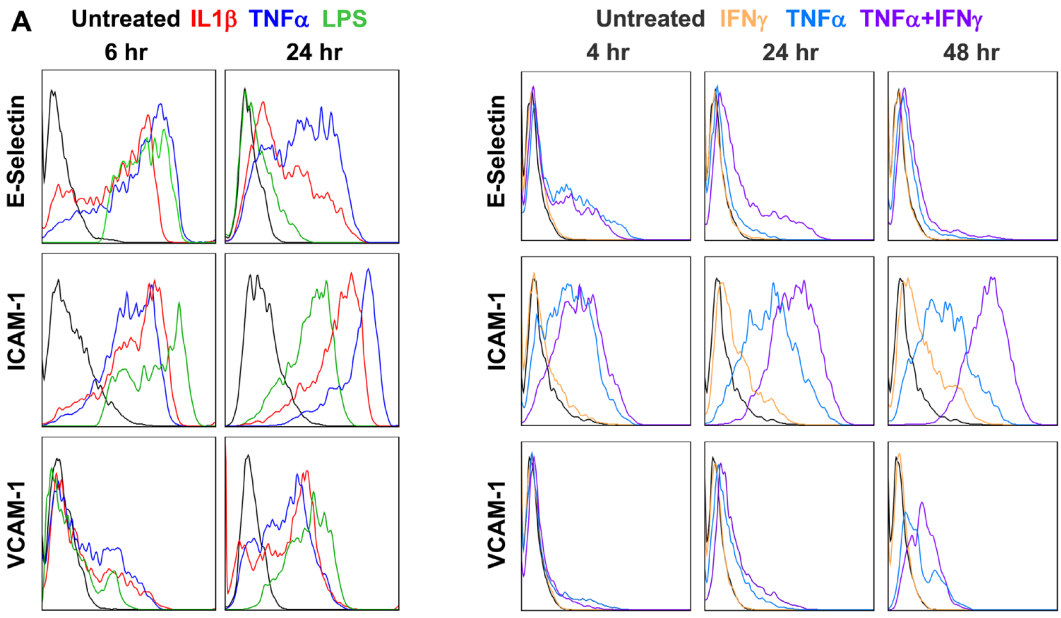

Figure 3.7 | (A) Surface expression of E-Selectin, ICAM-1 and VCAM-1 after 6 or 24 hr treatment with 10 U/mL IL1β, 10 ng/mL TNFα or 1 μg/mL LPS measured by flow cytometry. (B) Surface expression of E-Selectin, ICAM-1 and VCAM-1 after 4, 24 or 48 hr treatment with 10 U/mL IL1β and 10 ng/mL TNFα alone or in combination. Flow cytometry data are representative of three independent experiments.

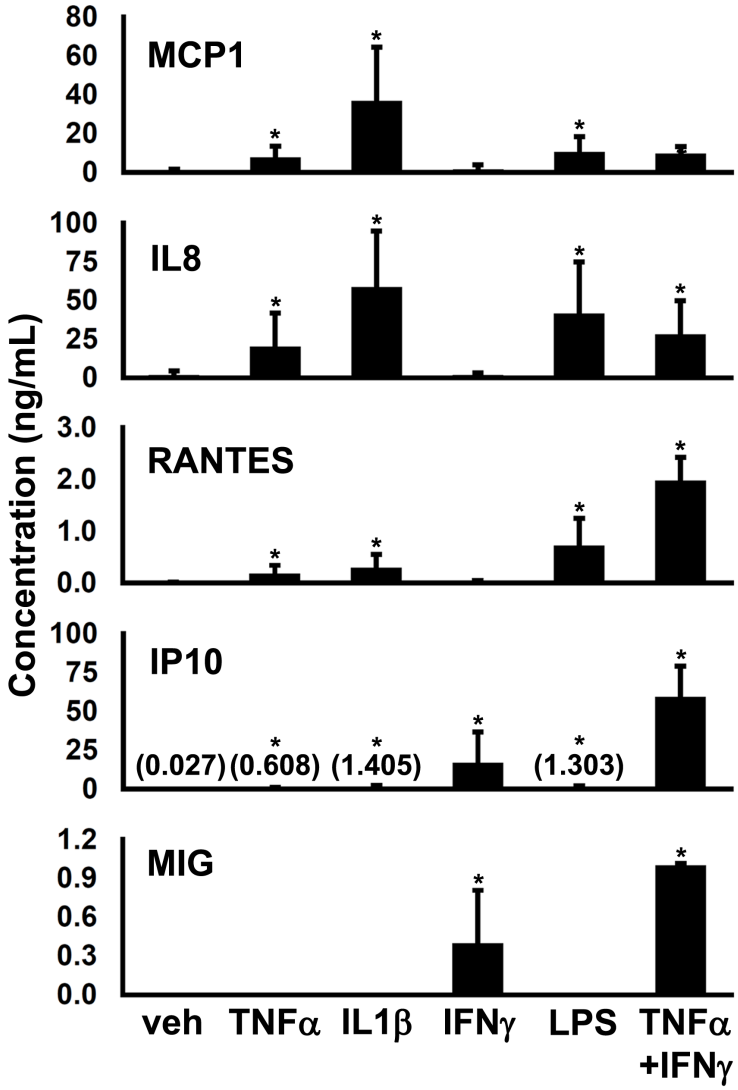

Figure 3.8 | Culture supernatant concentration of MCP1, IL8, RANTES, IP10 and MIG after 24 hr treatment of 10 ng/mL TNFα, 10 U/mL IL1β, 10 ng/mL IFNγ, 1 μg/mL LPS or 10 ng/mL TNFα + 10 ng/mL IFNγ measured by cytometric bead assay. Pooled data are represented as mean ± s.d. with * indicating statistical significance compared to vehicle tested by ANOVA with $p<0.05$, n=3-6.

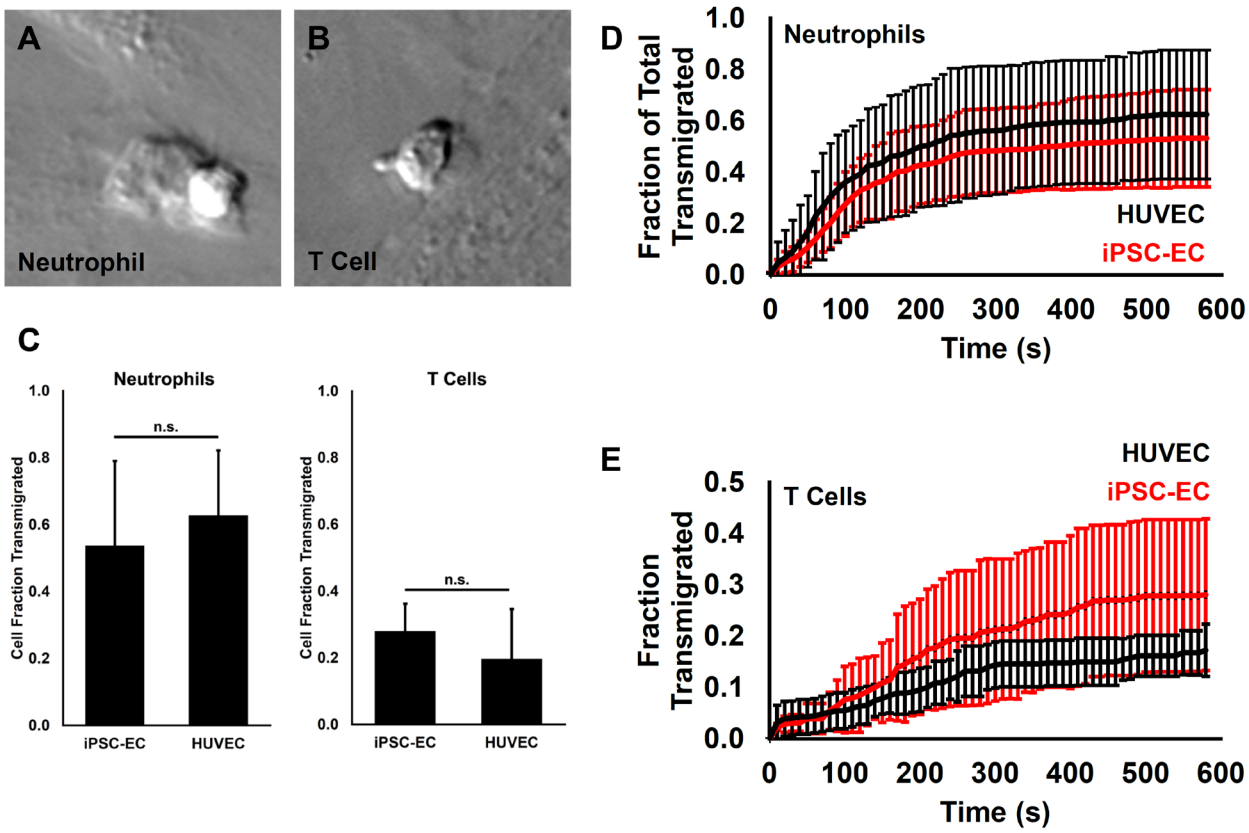

Figure 3.9 | Transmigration of a human (A) neutrophil and (B) T cell through a monolayer of iPSC endothelial cells. (C) Fraction of transmigrated neutrophils and T cells on HUVEC and iPSC endothelium after 10 min. Time profile of transmigrated (D) neutrophils and (E) T cells after 4 hr 10 ng/mL TNFα treatment. In all panels, pooled data are represented as mean ± s.d., n=3-4 independent experiments with three replicates for each experiment.

3.4 | Measurement of dynamic barrier response

An important function of the vascular endothelium is to form a tight dynamic barrier to contain the blood's plasma and cellular constituents. Endothelial cells actively rearrange junctional and cytoskeletal proteins to modulate their barrier function in response to multiple physiological stimuli[75,76]. To characterize the barrier phenotype of iPSC endothelial cells, I examined the role of known modulators of endothelial permeability on the electrical resistance across a monolayer of cultured iPSC endothelium, a real time measure of permeability. As seen in **Figure 3.10A**, the inflammatory mediator histamine induced a transient increase in permeability while VEGF induced a sustained increase in permeability. Prostaglandin E2, found at sites of inflammation, produced a decrease in permeability as did sphingosine-1-phosphate, which is produced by activated platelets. cAMP elevation and the subsequent activation of Epac proteins, which are cAMP-responsive guanine exchange factors for Rap GTPase, have been shown to decrease permeability of endothelial monolayers[77]. Treatment of iPSC endothelial cells with the cAMP analogue, 8-pCPT-2'O-Me-cAMP (O-Me) that selectively activates Epacs[77], induced a sustained decrease in permeability as reported in primary EC[78]. I next documented rearrangements of structural proteins that modulate barrier properties[76]. As seen in **Figure 3.10B**, I found that treatment with O-Me resulted in redistribution of VEC from a jagged to a more linear geometry and favored the generation of cortical actin over longitudinal stress fibers, as previously described in primary vascular endothelium[78]. These results suggest that iPSC derived endothelium may be a model system to study the effects of endothelial genetic contributions to vascular permeability.

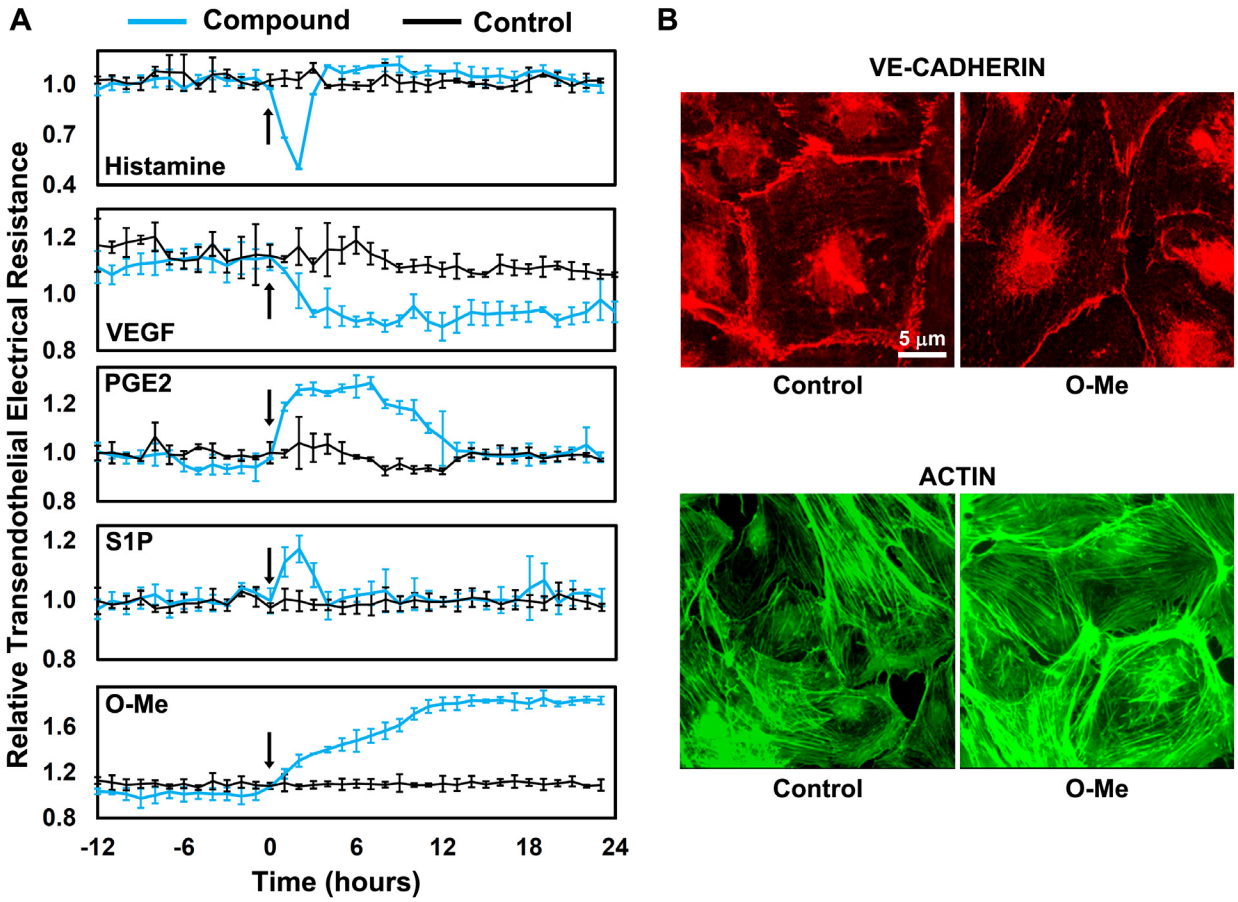

Figure 3.10 | (A) Changes in transendothelial electrical resistance after treatment with 10 µM histamine, 100 ng/mL VEGF, 200 ng/mL prostaglandin E2, 0.5 µg/mL sphingosine-1-phosphate or 100 µM O-Me. Traces are represented as mean ± s.d., n=3. (B) Rearrangements of VEC or actin after 24 hr treatment with 100 µM O-Me seen by immunofluorescence.

3.5 | Characterization of biomechanics-induced atheroprotected and atheroprone phenotypes

One of the exciting putative applications of iPSC derived vascular endothelium is the ability to model endothelial function and dysfunction in a patient specific manner. A fundamental aspect of endothelial function and dysfunction involves the endothelial response to mechanical stimulation providing by flowing blood, or hemodynamics. Interestingly, distinct local hemodynamic environments have been linked to the nonrandom predictable distribution of atherosclerotic lesions in human and animal studies[79-81]. In fact, local biomechanical forces can strongly influence endothelial phenotype and can induce a functional phenotype or one that is dysfunctional, a state seen as an early step in atherogenesis[82-85]. In particular, it has been previously shown that the specific shear stress patterns exerted on endothelial cells at the sites of atheroresistance, such as in the distal internal carotid artery, and atherosusceptibility, as in the carotid sinus in the human carotid artery induce "atheroprotective" and "atheroprone" cellular phenotypes respectively in cultured endothelial cells[86]. Therefore, I sought to determine whether similar biomechanical stimuli are able to evoke these characteristic biomechanically-induced phenotypes in iPSC endothelial cells. To investigate this, I exposed the cells to atheroprotective or atheroprone shear stress waveforms for 72 hr. As seen in **Figure 3.11A and B**, the endothelial cells selectively responded to the atheroprotective flow by aligning in the direction of flow while those exposed to atheroprone flow did not. Single periods of the atheroprotective and atheroprone shear stress waveforms derived from the sites of atheroresistance or atherosusceptibility in the human carotid are provided in **Figure 3.11C**. A key molecular descriptor of the endothelial atheroprotective versus atheroprone phenotype is the expression of the transcription factors Kruppel-like factor 2 (KLF2) and 4 (KLF4), integrators of the flow-mediated vasoprotective phenotype[87-90]. I measured the expression of these genes in iPSC endothelial cells exposed to atheroprotective or atheroprone shear stress waveforms and observed that the atheroprotective waveform differentially induced the expression of *KLF2* and *KLF4* (**Figure 3.11D**). Furthermore, I measured the expression of several other mechano-activated genes and downstream effectors of *KLF2* and *KLF4* and observed that atheroprotective shear stress upregulates *eNOS*, *argininosuccinate synthase 1*

(*ASS1*), and down-regulates *vWF*. In contrast, atheroprone flow increased the expression of *endothelin-1 (ET1)*, a potent vasoconstrictor as previously described to be differentially regulated by flow[87]. These data indicate that iPSC endothelial cells are able to respond to distinct biomechanical stimuli and adopt an atheroprotective or atheroprone phenotype. This recapitulated capability may enable new studies of patient specific endothelial function and dysfunction relevant to atherogenesis.

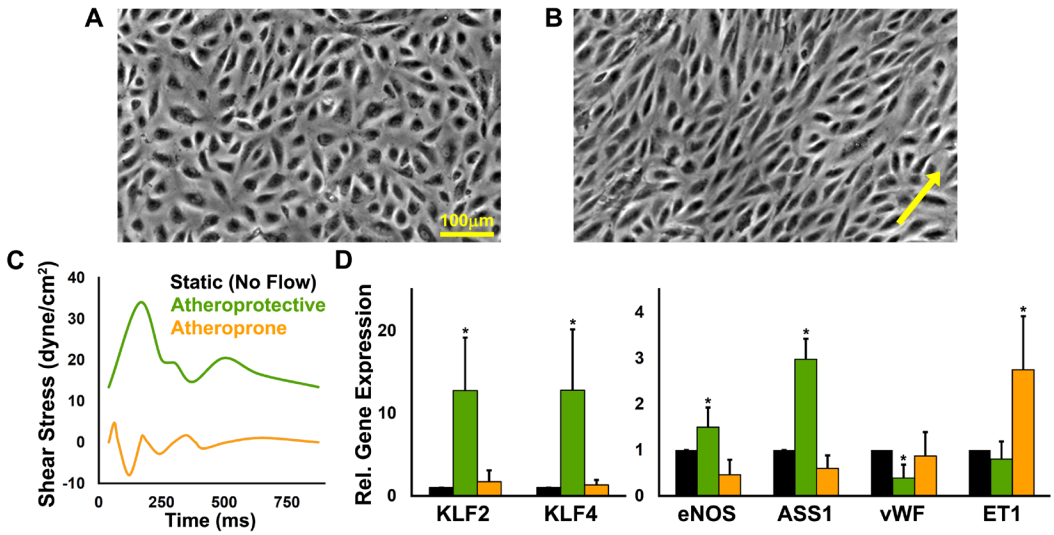

Figure 3.11 | . iPSC endothelial cells seen by phase contrast after 72 hr of (A) atheroprone or (B) atheroprotective flow (arrow indicates direction of flow). (C) A single period of atheroprotective and atheroprone shear stress waveforms. (D) 72 hr of atheroprotective shear stress upregulates gene expression of *KLF2*, *KLF4*, *eNOS*, *ASS1*, and downregulates *vWF* as atheroprone shear stress upregulates *ET1*. Pooled data are normalized to expression under static conditions and represented as mean ± s.d. with * indicating statistical significance with p<0.05, n=4.

3.6 | Evaluation of pharmacologically induced atheroprotection

An exciting potential application of iPSC derived vascular endothelium is their ability to serve as a personalized substrate for pharmacological testing. It is known that endothelial cell phenotype is sensitive to several widely used pharmacological agents such as cyclooxygenase-2 inhibitors, PPAR modulators and statins[91,92]. I sought to examine whether iPSC endothelium is similarly sensitive to one such drug to indicate whether iPSC endothelial cells may be a surrogate to study drug induced vascular effects. Specifically, it has been documented that the expression of KLF2 and an endothelial atheroprotective phenotype can be evoked by a pleiotropic effect of the statins, a class of HMG-CoA reductase inhibitors[93,94]. Therefore, I examined whether iPSC endothelial cell phenotype can be similarly modulated by statin treatment. As seen in **Figure 3.12**, simvastatin upregulated atheroprotective *KLF2* expression as well as several downstream effectors such as *eNOS*, *ASS1*, *thrombomodulin (TM), integrin beta 4 (INTB4)*, *prostaglandin-H2 D-isomerase (PTGDS)*. Simvastatin also led to the down-regulation of pro-inflammatory *angiopoietin-2* (*ANGPT2*) and vasoconstrictor *ET1*. Furthermore, the induction of *KLF2* and *eNOS* in iPSC derived endothelial cells showed similar sensitivity to simvastatin concentration (1-10 μM) as primary endothelial cells (**Figure 3.13**). This data suggests that iPSC derived endothelium serves as a faithful model of at least the endothelial atheroprotective cellular phenotype induced by statins.

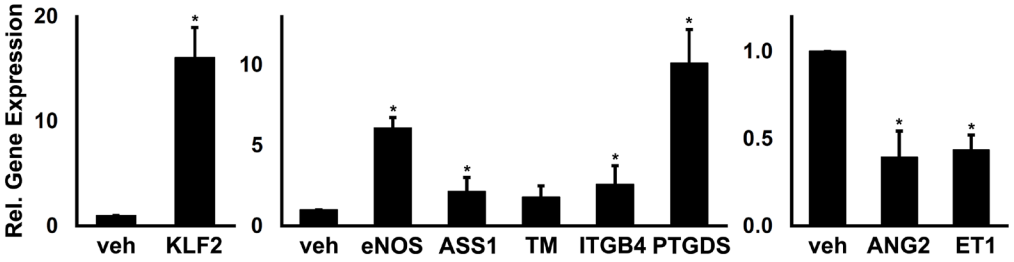

Figure 3.12 | (A) Induction of atheroprotective genes and down-regulation of atheroprone genes in iPSC endothelial cells in response to 24 hr of 10μM simvastatin measured by qRT-PCR. Data are normalized to expression in response to vehicle and plotted with error bars equal to s.d. * indicates significant statistical difference from vehicle with $p < 0.05$, n=4.

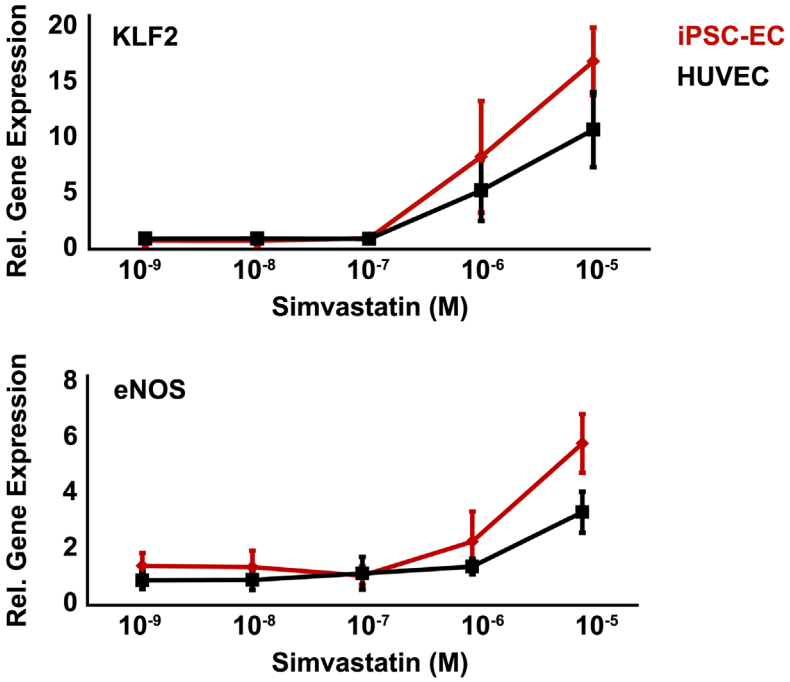

Figure 3.13 | (A) Induction of *KLF2* and *eNOS* gene expression measured by qRT-PCR in response to 24 hr treatment with varying doses of simvastatin in HUVEC and iPSC endothelial cells. Data are represented as means ± s.d., n=4-6.

4 | Arterial and venous identities within human induced pluripotent stem cell derived vascular endothelium

Human vascular endothelium is a heterogeneous set collection of distinct cellular subtypes. These vascular endothelial identities vary by their anatomical location such as in the context of arterial, venous or capillary endothelium, residence in micro, small, or large vessels, or association with other organs such as the glial cells in blood brain barrier endothelium, hepatocytes in liver sinusoidal endothelium or podocytes in glomerular endothelium. In the previous experiments, I considered the iPSC derived vascular endothelium as a single population of undefined identity; however, in the following experiments, I sought to evaluate whether the iPSC derived vascular endothelium could be directed to an arterial or venous fate and whether there was any existing molecular heterogeneity from the embryoid body derived cells suggesting an arterial or venous identity. This line of investigation is essential for future applications of iPSC where genetic diseases manifest selectively in arterial or venous endothelium, or for cell-based assays of endothelial function which is known to vary between different endothelial subtypes.

4.1 | Biomechanical specification to arterial and venous identities

There are numerous lines of evidence indicating that the local biomechanical environment of vascular endothelial cells plays an important role in establishing the arterial or venous fate of the endothelium as elaborated in Section 1.2. I wished to examine whether iPSC derived vascular endothelium could similarly display an arterial or venous identity sensitive to its biomechanical environment. To assess this, I applied two different shear stress waveforms to the cultured iPSC endothelial cells; one derived from the shear stress applied to the wall of the human abdominal aorta and one from the human saphenous vein. These arterial and venous waveforms, of which individual periods are plotted in **Figure 4.1A**, were applied to the cells for 72 hr. After these conditions, I assayed the

identity of the endothelial cells by measuring the expression of a panel of genes which are differentially expressed on arterial and venous endothelial cells. I observed that the arterial shear stress condition upregulated expression of *notch4*, *hey1*, *hey2*, *ephrinB2* and *ephB4*, *endomucin* and *VEGF* and down-regulated expression of *delta-like ligand-1*, *jagged1*, *angiopoietin-2*, and *vWF*. Alternatively, venous shear stress upregulated expression of EphB4 and endomucin. These gene expression signatures[30-33] suggest that arterial and venous shear stress environments *in vitro* are able to guide iPSC endothelium towards an arterial and venous fate, respectively. Interestingly, endomucin, which is typically expressed to a greater extent on venous endothelium than arterial endothelium[95], is upregulated by both arterial and venous shear stress in cultured iPSC endothelium. It is also interesting to note that arterial flow down-regulated expression of *vWF*, which is known to be expressed to a greater degree on venous endothelium[96] and also known to contribute to atherogenesis in a hemodynamic-environment dependent manner[97]. These results suggest that iPSC derived vascular endothelium exhibits an identity sensitive to biomechanical stimulation which is able to be guided to arterial or venous fate as indicated by gene expression by different flow waveforms.

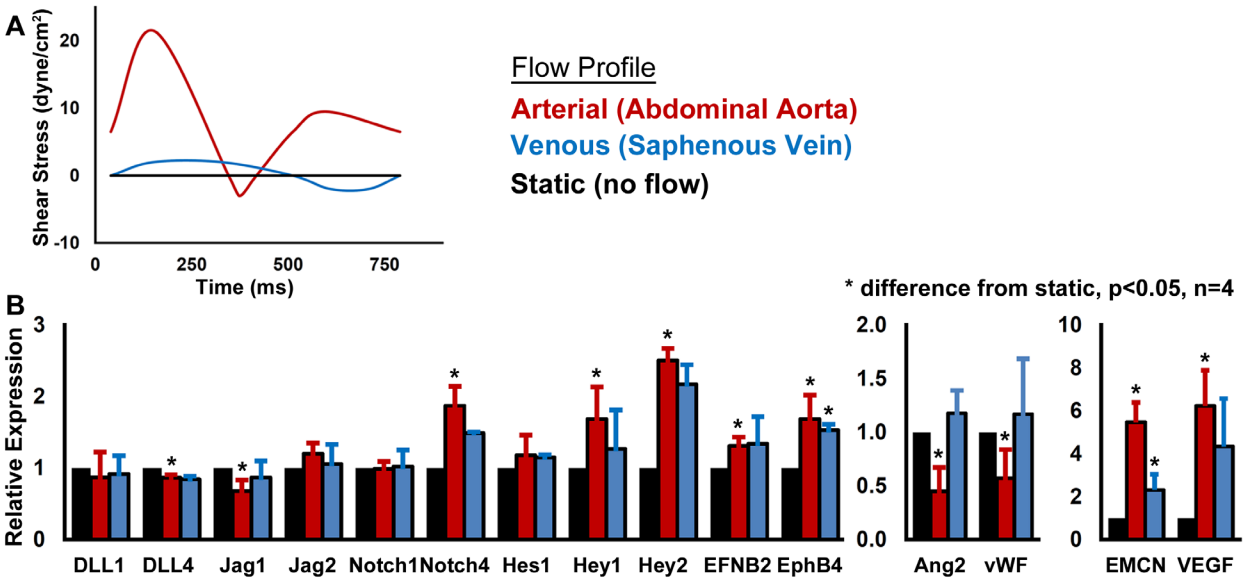

Figure 4.1 | (A) A single period of arterial and venous shear stress waveforms. (B) Gene expression of arterial and venous markers in iPSC hEC. Pooled data are represented as means ± s.d., n=4. * indicates p<0.05 comparing expression of each gene to the static no flow condition.

4.2 | Genetic specification to arterial and venous identities

During embryonic development, arteries and veins form lined with vascular endothelial cells with distinct molecular identities, as mentioned in section 1.2. Despite the influence of biomechanical forces on arterial and venous identity, the initial adoption of arterial and venous fate occurs prior to the onset of circulation in the developing organism[43,45]. This observation suggests that developmental pathways specified by genetic programs are sufficient to establish unique arterial and venous endothelial subtypes. Thus, if genetic specification alone is able to give rise to distinct vascular endothelial subtypes during development in the absence of hemodynamic environments, it is possible that pluripotent stem cells are also able to differentiate into arterial and venous endothelial cells *in vitro*. Hence, I investigated whether the iPSC derived differentiating embryoid bodies can also produce arterial and venous subtypes of vascular endothelial cells as diagramed in **Figure 4.2**.

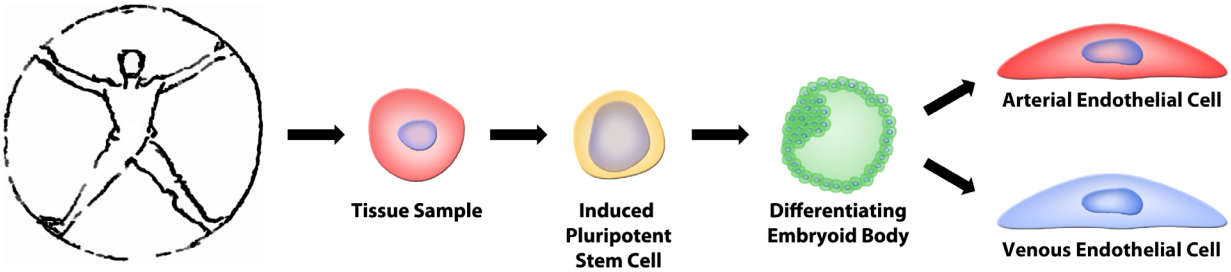

Figure 4.2 | Illustration of the process of generating subtype specific endothelial cells from patient tissue samples via induced pluripotent stem cells.

To assess this, it is necessary to separate arterial and venous endothelial cells from the heterogeneous embryoid bodies. Extensive previous work has identified the molecular signatures that define arterial and venous endothelial cells (as previously reported[30-33]). The protein and gene expression constituting such signatures are generally not mutually exclusive, i.e. both arterial and venous endothelial cells tend to express the same markers though to differing magnitudes. However, the best characterized marker of arterial fate, the transmembrane ligand ephrinB2, is exclusively expressed on arterial endothelial cells and absent in venous endothelial cells in both embryonic[44] and post-natal[98] vasculature.

The cognate receptor for ephrinB2, ephB4 is exclusively expressed on venous endothelial cells during embryonic development; however this exclusivity is lost during development as both arterial and venous endothelial cells gain ephB4 expression[44,98]. A potential strategy to separately isolate arterial and venous endothelial cells from the embryoid body would be to sort cells expressing VE-Cadherin and also sort the ephrinB2 positive and negative populations which would represent arterial and venous endothelial cells respectively. However, flow cytometry was not able to resolve ephrinB2 expression using two different anti-ephrinB2 antibodies. The lack of high quality antibodies against ephrinB2 has been commented on previously[99]. Other groups have identified the chemokine receptor CXCR4 as another surface marker of arterial endothelial cells. CXCR4 has been shown to be selectively present in arterial vasculature as in zebrafish anterior (but not posterior) lateral dorsal aortae[100], in the murine dorsal aorta but absent in the cardinal veins of the aorto-gonado-mesonerphros region of the mouse embryo[101], in the developing mesenteric arteries of the small intestine also in the mouse embryo[102] as well as in the developing arterioles of murine skin[103]. Studies in the zebrafish have shown that once connection with the arterial network and perfusion through new arteries commences that CXCR4 expression is lost[104]. This is corroborated by the fact that shear stress applied to cultured endothelial cells also downregulates CXCR4 expression[105]. Generally, it is believed that interaction between CXCR4 and its ligand CXCL12 (also known as SDF-1) contributes to vessel patterning[106]. Previously, other groups have used CXCR4 as a marker of arterial endothelial cells when segregating embryoid bodies derived from mouse embryonic stem cells[101,107]. Thus, I examined expression of CXCR4 within the human iPSC derived differentiating embryoid bodies. When examining CXCR4 and VEC expression in dissociated embryoid bodies, it is clear that there are four populations (**Figure 4.3, left panel**). When visualizing only the VE-Cadherin expressing cells, a VE-Cadherin+|CXCR4+ population is resolved (**Figure 4.3, center panel**). After repeating this observation, rather consistently 35% ± 3% (mean ± s.d.) of the endothelial cells within the embryoid body express CXCR4 and are likely of arterial identity (**Figure 4.3, right panel**).

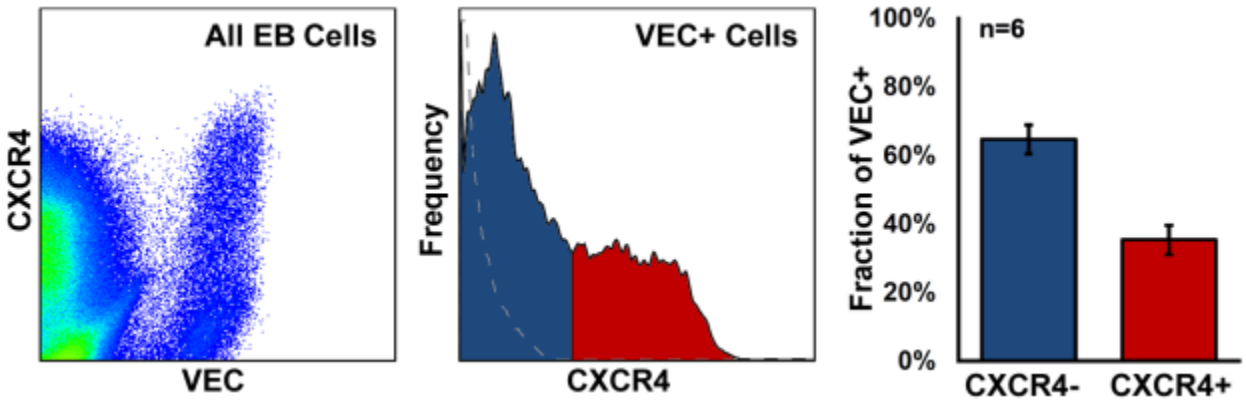

Figure 4.3 | Flow cytometry analysis of CXCR4 and VE-Cadherin expression showing VEC+|CXCR4+ and VEC+|CXCR4- populations (left and center, dotted trace is a matching isotype control). The fraction of VEC+ cells which express CXCR4 is reproducible (right). Flow cytometry plots are representative. Pooled data are represented as means ± s.d., n=6.

Following observation that there exists a reproducible subpopuation of embryoid body derived endothelial cells expressing CXCR4, I wished to evaluate whether this subpopulation more closely resembles arterial endothelium as would be expected from published data. To do this, I used fluorescence assisted cell sorting to isolate live VEC+ and CXCR4+ or CXCR4- cells from embryoid bodies and measured the expression of a panel of genes associated with arterial identity (**Figure 4.4**). The CXCR4+ subfraction of VEC+ endothelial cells expressed statistically significant greater ephrinB2, hey2, notch4, connexin-40 (Cx40, also known as ZO-1), connexin-37 (Cx37) and tissue plasminogen activator (tPA). As expected, there was no significant differnece in expression of VEC or β-actin between the CXCR4± subfractions. Interestingly, there also was no significant difference in the expression of vWF, despite the fact that it is known to be differentially expressed in arterial and venous endothelium as mentioned above. These results suggest that there does exist molecular heterogeneity among the VEC+ endothelial cells derived from iPSC with arterial and venous identity polarized along an axis of expression of CXCR4. It is noteworthy that this molecular distinction between these arterial and venous subtypes exists in the complete absence of any biomechanical stimulation in the history of these endothelial cells, a feature not

shared with other *in vitro* models of endothelial cell biology where cells are derived from vessels which have harbored blood flow at some point in their natural history.

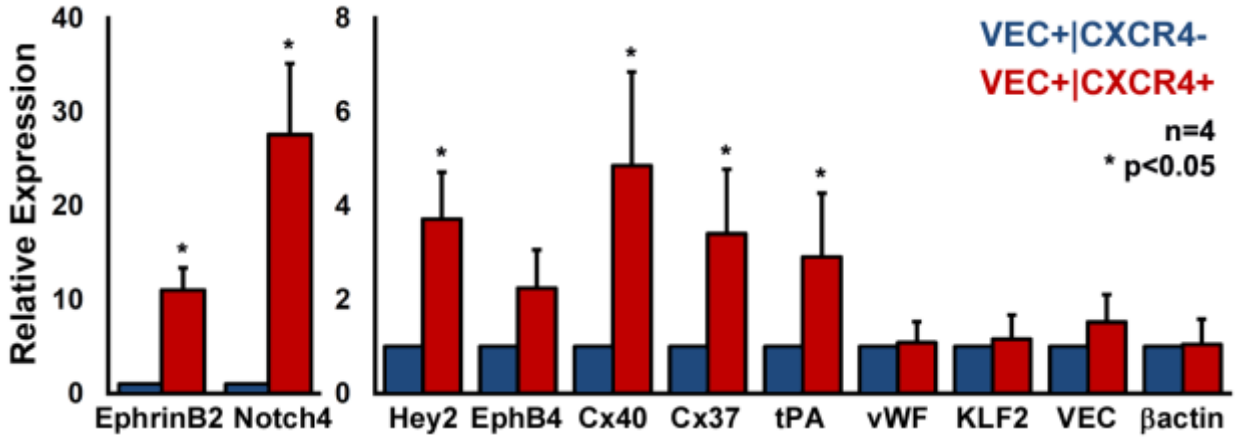

Figure 4.4 | Expression of genes in VEC+|CXCR4+ and VEC+|CXCR4- populations. Pooled data are represented as means ± s.d., n=4. * indicates p<0.05 comparing expression of each gene between populations.

4.3 | Assessing the origin of the arterial/venous differential expression of vWF

Due to the preponderance of evidence that arterial and venous identities are guided by genetic programs during embryonic development but with sufficient plasticity to be sensitive to their local hemodynamic environment, it is likely that some attributes of the mature arterial and venous identity may be attributable to their genetic ontogeny, others to the biomechanical environment and some responsive to both. Endothelium derived from iPSC offer a unique model system of endothelial cell development as the first onset of hemodynamic stimuli can be experimentally controlled. In particular, these results regarding arterial and venous identity (**Figure 4.4**) show that while there is a clear molecular distinction between the arterial CXCR4+ and venous CXCR4- subsets of endothelial cells in terms of expression of arterial specific genes ephrinB2, notch4, hey2, cx40, cx37, and tPA there is no differential expression of vWF in contrast to what is expected based upon *in vivo* observations. However, arterial biomechanical stimulation applied to the pooled CXCR4± populations induced a down-regulation of vWF, reconstituting the subtype specific expression pattern (**Figure 4.1**). This suggests that the differential expression of vWF observed in arterial and venous endothelial cells is a consequence of their biomechanical environments rather than their genetic ontogeny. To better define this process and to evaluate whether one of the CXCR+ or CXCR4- subsets is specifically responding to the arterial shear stress, I sought to perform a similar experiment but with isolated purified VEC+|CXCR4+ and VEC+|CXCR4- populations independently. Purification of these populations based on expression of two cell surface markers presented a new challenge as the magnetic bead based purification methodology is only able to positively select cells in a binary fashion based on a single molecular criterion. To address this, I experimented with a commercial product offering enzymatic dissociation magnetic bead conjugated antibodies (Miltenyi) with the goal of performing two serial magnetic bead based positive selection sorts. However, sorting with this system, either for VEC then CXCR4 or vice versa, produced low yields and poor purity in the final isolated populations (<50%). The performance of this methodology is clearly not viable for downstream applications. Following this, I turned to sorting with fluorescence based cell sorting (FACS). At the outset

of the iPSC endothelial cell isolation experiments described in Section 2.2. I attempted to isolate the VEC+ cells with FACS following embryoid body dissociation. However, in comparison to magnetic bead sorting, FACS isolation produced poor survival leading me to use magnetic bead based separation for the experiments described in Section 3. Now, with the necessity of sorting based on two antigens, I altered some of the machine parameters of the FACS isolation, opting for an increased size of nozzle from 70μm to 100μm, slowing the fluid stream velocity, lowering the sheath pressure, minimizing vortexing and maintaining the cell slurry at 4°C. These conditions significantly improved survival and permitted isolation of monolayers of VEC+|CXCR4± populations as seen in **Figure 4.5**.

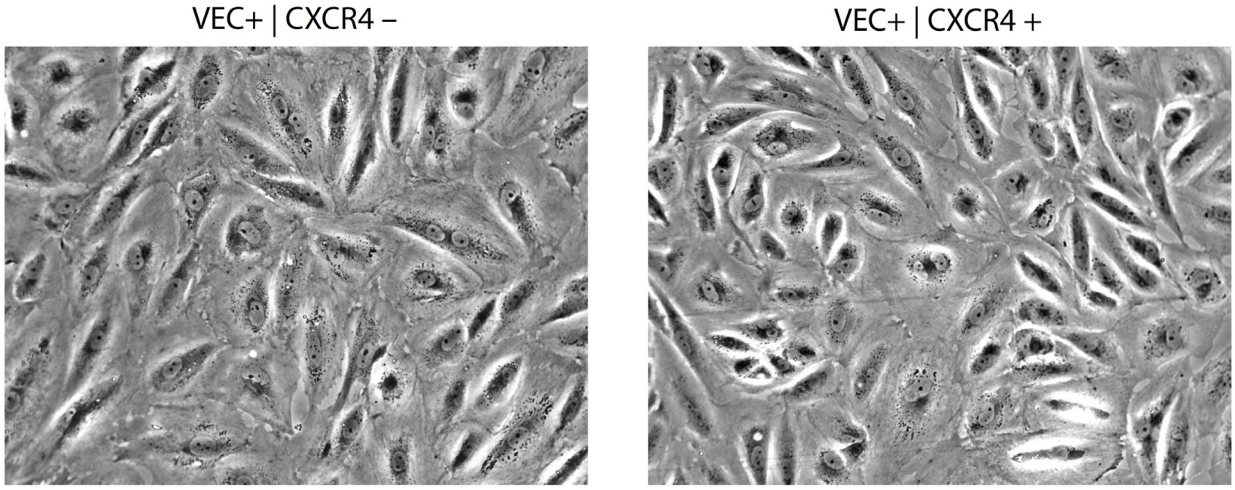

Figure 4.5 | Phase contrast images of the CXCR4+ and CXCR4- purified subsets of VEC+ iPSC endothelial cells after FACS isolation.

Using these conditions, I sought to use purified isolated VEC+|CXCR4+ and VEC+|CXCR4- populations and independently examine the expression of vWF in each population after exposure to arterial and venous shear stress regiments or static conditions. This experiment is schematically outlined in **Figure 4.6**, where VEC+|CXCR4+ and VEC+|CXCR4- endothelial cell populations are isolated from embryoid bodies by FACS, then cultured independently under static conditions for 3 days allowing

formation of a confluent monolayer, and then exposed to 3 days of either arterial, venous or static flow conditions *in vitro* as previously shown in **Figure 4.1**. Following the arterial biomechanical stimulation, it was apparent that both VEC+|CXCR4+ and VEC+|CXCR4- endothelial cell populations elongated and aligned in the direction of flow as seen in **Figure 4.7**. I also observed that arterial biomechanical stimulation resulted in about half the magnitude of vWF gene expression as observed after static or venous shear stress. This arterial shear stress mediated down-regulation of vWF expression was observed to an equal degree in both VEC+|CXCR4+ and VEC+|CXCR4- endothelial cell populations. These observations suggest that the biomechanical environment, not the genetic ontogeny, is responsible for the differences in vWF expression observed in the vascular tree.

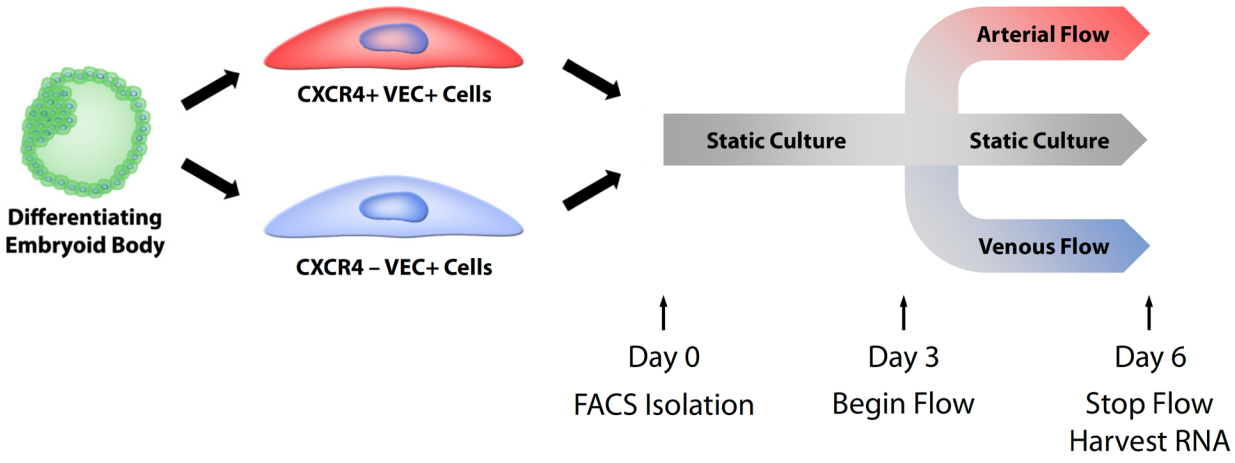

Figure 4.6 | Schematic representation of the experiment involving the FACS isolation of VEC+|CXCR4± cells, static culture for 3 days and subsequent exposure to arterial, venous or static conditions for another 3 days after which RNA is harvested.

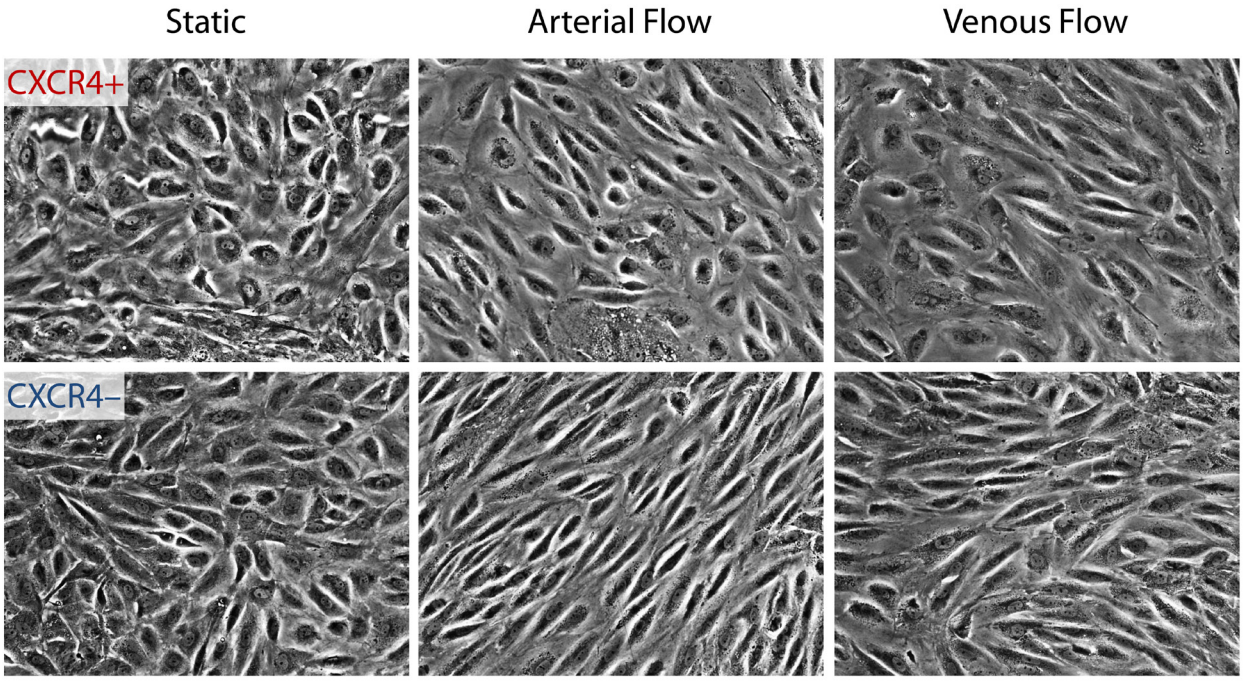

Figure 4.7 | Phase contrast images of CXCR4+/- hEC after static, arterial flow or venous flow conditions.

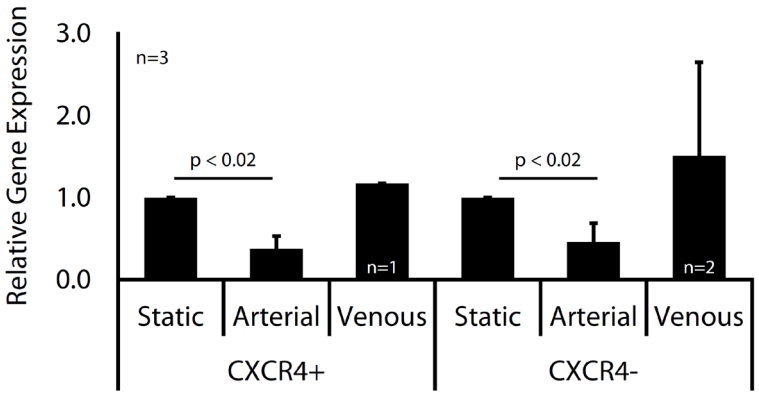

Figure 4.8 | Gene expression of vWF in CXCR4+/- hEC after static, arterial or venous flow conditions.

5 | Methods

5.1 | Human induced pluripotent stem cell culture and differentiation

Human iPSC were cultured as previously reported[108], on irradiated mouse embryonic fibroblasts (GlobalStem, Rockville, MD) and passaged mechanically. Four human iPSC lines were used. Lines BJ1 and MSC1 were received from the laboratory of Dr. George Q. Daley (Children's Hospital Boston), line BMC1 in the laboratory of Dr. Darrell N. Kotton (Boston University Medical Center) and line DH1F from the Harvard Stem Cell Institute. All lines were created by overexpression of reprogramming genes *OCT4*, *SOX2*, *KLF4* and *CMYC* though reprogrammed through different technologies. Line BJ1 was created with 4 retroviruses from human dermal fibroblasts[109]. MSC1 was created with 4 retroviruses from human mesenchymal stem cells[109]. BMC1 was created with a single lentivirus from human dermal fibroblasts[110]. DH1F was created with modified-RNAs from human dermal fibroblasts[111]. The BJ1 iPSC line was used for detailed functional characterization of the derived EC.

Human iPSC medium consisted of DMEM:F12 (Invitrogen, Life Technologies, Carlsbad, CA), 20% Knock Out Serum Replacement (Invitrogen), 5 ng/mL bFGF (Miltenyi, Bergisch Gladbach, Germany), 110 μM 2-mercaptoethanol (Sigma, St. Louis, MO), 100 μM non-essential amino acids (Invitrogen), 50 ng/mL ascorbic acid (Sigma), and 125 U/mL penicillin and 125 μg/mL streptomycin (Lonza, Basel, Switzerland). Differentiation medium consisted of DMEM, 20% fetal calf serum (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine (Invitrogen), 110 μM 2-mercaptoethanol (Sigma), 100 μM non-essential amino acids (Invitrogen), 50 ng/mL ascorbic acid (Sigma), and 125 U/mL penicillin and 125 μg/mL streptomycin (Lonza). Endothelial cell medium consisted of M199 (Lonza), 20% fetal calf serum, 2 mM L-glutamine, 50 μg/mL EC growth supplement (Sigma), 100 μg/mL heparin

(Sigma), 100 μM non-essential amino acid solution, and 125 U/mL penicillin and 125 μg/mL streptomycin.

5.2 | Culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords, pooled, and cultured as described previously[93] by the cell culture core laboratory of the Center for Excellence in Vascular Biology at the Brigham and Women's Hospital.

5.3 | Transmission electron microscopy and immunogold labeling

Samples were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde for 1 hr and were processed by the Harvard Medical School Electron Microscopy Facility. Samples were examined on a TecnaiG2 Spirit BioTWIN mission electron microscope with images recorded with an AMT 2k CCD camera. Immunogold labeling used a rabbit anti-human vWF antibody (#A008202, Dako, Glostrup, Denmark) with protein A conjugated to gold particles of 25 nm diameter.

5.4 | Biomechanical stimulation with shear stress *in vitro*

In the course of the experiments mentioned above, various biomechanical stimulation regiments were used. Specifically, time-varying waveforms replicating the shear stress present on the endothelial surface within the distal human internal carotid artery ("atheroprotective"), carotid sinus ("atheroprone"), abdominal aorta ("arterial") or saphenous vein ("venous") were measured from human subjects and applied to iPSC-EC using a custom cone and plate dynamic flow system as reported[86,112].

5.5 | Transendothelial migration of leukocytes

Human neutrophils and T cells were isolated as previously reported[113]. The interaction of leukocytes and iPSC-EC was assessed in a custom flow chamber as previously reported[113]. Briefly, iPSC-EC were treated with 25 ng/mL TNFα for 4 hr prior to both transmigration assays and additionally 50 ng/mL SDF-1α for 15 min prior to the T cell transmigration assay.

5.6 | Measurement of endothelial permeability properties

Measurements of electrical resistance across the EC monolayer were performed as previously reported[78] with an impedance sensor system (Applied Biosystems, Life Technologies, Carlesbad, CA). Data were normalized as the ratio of measured to baseline resistance.

5.7 | *In vitro* angiogenesis assay

The *in vitro* angiogenesis assay was performed as previously described[114]. Briefly, basement membrane extract (Trevigen, Gaithersburg, MD) was incubated on multiwell culture plates for 1 hour at 37°C after which iPSC derived endothelial cells were seeded at 20,000 cells/cm^2 then observed after 6 hr of culture. Cells were grown in EC medium with reduced fetal bovine serum (2%) and reduced endothelial cell growth supplement (2.5 μg/mL).

5.8 | RNA extraction, isolation and quantitative real-time PCR

Total RNA was extracted using the Nucleic Acid Purification Lysis Solution, isolated with 6100 Nucleic Acid PrepStation and eluted into 80μL and then reverse transcribed into cDNA with a High

Capacity RNA to cDNA Kit in a Verite thermocycler, all according to the manufacturer's (Applied Biosystems) protocols. Quantitative TaqMan real time polymerase chain reactions (qRT-PCR) were performed in 10μL in 384-well format on a 7900HT sequence detection system (Applied Biosystems) with TaqMan primers and fluorescent probes provided by Applied Biosystems.

5.9 | Immunofluorescence and imaging

Endothelial cells in culture were washed with warmed PBS+, fixed in warmed 4% paraformaldehyde for 5 min, permeabilized in 0.5% Triton X-100 in PBS for 5 mins, blocked with PBS+ with 15% fetal bovine serum for 30 mins and then incubated with primary antibody for 2 hr at room temperature. After three washes with PBS they were incubated with secondary antibodies for 1 hr at room temperature. Immunofluorescent labeled cells were visualized on a Nikon TE2000 fluorescence microscope and captured with a Hamamatsu CCD camera. Mouse anti human primary antibodies against eNOS (# 610296 BD Biosciences), VE-Cadherin (#560410 BD Biosciences), CD31 (#555445 BD Biosciences), and a rabbit anti human vWF (#A008202, Dako), angiopoietin-2 (#AF623 R&D Systems) were used. Goat anti-mouse or rabbit Alexa 488 and Alexa 546 were used as secondary antibodies (Invitrogen).

P-selectin surface expression was assessed similarly though without permeabilization, using a mouse anti-human P-selectin antibody (#555523 BD Biosciences, San Jose, CA) and fluorescence plate reader (M2 Spectramax, Molecular Devices, Sunnyvale, CA). Fluorescent Dil-acetylated-LDL was purchased from Invitrogen. Actin was stained with FITC-phalloidin (Invitrogen). Nuclei were stained with DAPI at 10 ng/mL (Sigma).

Whole embryoid bodies were immunofluorescently labeled by gently washing with warm PBS+, fixing in warmed 4% paraformaldehyde for 10 minutes, and washing 3 times in PBS+ with 15% fetal

bovine serum. The EBs were incubated with primary antibodies against VE-Cadherin and CXCR4 for two hours at room temperature then washed 3 times. Following this, they were incubated in secondary antibodies and DAPI for 1 hour at room temperature then washed 3 times. After mounting on slides, EBs were visualized with a Zeiss 710 laser scanning confocal multi-photon system (Zeiss, Oberkochen, Germany).

5.10 | Flow cytometry analysis and sorting.

Cells were lifted from their substrate with Cell Dissociation Buffer (Invitrogen) and blocked with PBS containing 10% fetal bovine serum for 20 minutes on ice. Primary antibodies were added to the blocking solution for 20 min on ice. Cells were washed and incubated with secondary antibodies if necessary for 20 min on ice. After washing, labeled cells were measured by flow cytometry on a BD FACSCalibur and analyzed by Flowjo software (Tree Star, Ashland, OR). Mouse IgG1 primary antibodies against human VE-Cadherin (FITC or PE conjugated, #560410, #560411 BD Biosciences), CD31 (FITC conjugated, #555445 BD Biosciences), CXCR4 (PE conjugated #555947 BD Biosciences), KDR (PE conjugated #560494 BD Biosciences), and thrombomodulin (PE conjugated #559781 BD Biosciences) were purchased along with appropriate isotype controls. Mouse IgG1 antibodies against human E-selectin (H 4/18), ICAM-1 (Hu 5/2) and VCAM-1 (E 1/6) or isotype control (K 16/16) were a generous gift of the laboratory of Dr. Michael A Gimbrone Jr and were used with goat anti mouse Alexa 488 (Invitrogen). To measure the extracellular glycocalyx glycoproteins and carriers, flow cytometry was performed with antibodies raised against syndecan-1 (biotin conjugated #ab27362, Abcam, Cambridge, MA), syndecan-2 (APC conjugated #FAB2965A, R&D Systems, Minneapolis, MN), syndecan-4 (biotin conjugated #BAF2918 Abcam), glypican-1 (biotin conjugated #BAF4519, R&D Systems), CD44 (biotin conjugated #555477 BD Biosciences), heparan sulfate (#H1890, US Biological, Salem, MA) or chondroitin sulfate (#554275 BD Biosciences). Alexa 488 goat anti mouse IgG2a, or goat anti mouse IgM or streptavidin-conjugated Alexa488 (Invitrogen) were used as secondary antibodies as appropriate.

5.11 | Measurement of soluble cytokines with cytometric bead assay

Concentrations of soluble IL8, MCP1, RANTES, IP10 and MIG in cell culture supernatants were measured using a cytometric bead assay (BD Biosciences) according to the manufacturer's instructions and read on a FACSCalibur flow cytometer (BD Biosciences) after appropriate fluorescent calibration and compensation using manufacturer provided beads. Calibration, compensation, measurement of standard curves for concentrations from 10 pg/mL to 2500 pg/mL and fresh preparation of standards were performed at each experiment. Cytometric bead assay data were analyzed using Flowjo and Excel software.

5.12 | Statistical analysis

Statistical comparisons were performed using the two-sided non-parametric Mann-Whitney test or the two-sided unpaired T Test, if indicated, with Stata software. All pooled data in figures and in text are represented as the mean ± standard deviation. For multiple statistical comparisons, the one way analysis of variance test with Bonferroni correction was used. On figures, * indicates statistical significance with $p<0.05$. Flow cytometry and immunofluorescence data are representative of at least three experiments.

6 | Conclusions

6.1 | A new source of human vascular endothelium

In one of the initial reports describing the isolation of primary human endothelial cells in 1974, Michael A. Gimbrone Jr. and colleagues remarked[5] that "confluent endothelial cell cultures could be useful in studying the pathophysiology of endothelial growth." This introductory report heralded a prolific era of new studies discerning of the role of the vascular endothelial compartment in human pathophysiology. It is from these ensuing studies that the scientific community has developed its current knowledge of the vascular endothelium as a critical member of a distributed organ system, forming a single-cell layer lining the luminal face of blood vessels. This system constitutes a multifunctional interface with phenotypic plasticity that maintains vascular homeostasis, participates in inflammatory responses, triggers thrombosis, regulates vasomotor tone, controls vascular permeability and forms new vasculature when demanded.

Despite the significant progress in defining the pathophysiological role of vascular endothelium, human vascular endothelium used in research studies today remains to be sourced from discarded pathological, cadaveric or umbilical vessels. The procurement of vascular endothelial cells from such sources typically precludes the choice of donor patient and thus genetic background, limiting studies of the genotype-phenotype relationship to probe endothelial cell biology in the absence of non-physiological genetic manipulation. It would take new scientific advances from the field of embryology to provide the technological means to study *in vitro* vascular endothelial cells with specific genetic backgrounds.

After another seminal discovery, the first report detailing *in vitro* culture of mouse embryonic stem cells[6], Gail Martin contemporaneously noted[7] that "the availability of such [pluripotent] cell lines should make possible new approaches to the study of early mammalian development." The development of embryonic stem cells and the ensuing evolution of knowledge regarding the differentiation of

pluripotent cells into mature cell types introduced a new era for cell biology. Though Martin referred to the ability to study development in the context of particular genetic mutations, the differentiation of pluripotent cells into mature cell types also established a framework to study human development *ex vivo*, in particular in the absence of the complex milieu of biochemical and biomechanical stimulation inherent in the embryo. The repercussions of embryonic stem cell biology stretched beyond the study of embryogenesis and developmental programs as embryonic stem cells allowed the study cell types such as neurons and cardiac myocytes in the human system previously difficult to procure. While embryonic stem cells, first in the mouse, then the human, permitted the generation of vascular endothelium in the laboratory independent of securing particular patient donors, the embryonic stem cell system still lacked the ability to precisely control the genetic backgrounds of the cell lines. For some time, this would remain an impediment to genotype-phenotype studies of vascular development and endothelial cell biology as well as the development of new regenerative and personalized therapies.

This impediment was eventually overcome as new stem cell engineering methods were discovered with the ability to create all the diverse mature cell types as can be differentiated from embryonic stem cells though with the ability to begin with arbitrary patient donor samples and hence genetic backgrounds. The relatively recent discovery of human induced pluripotent stem cells by Shinya Yamanaka[11] effectively democratized the types of research of envisioned forty years ago by Evans, Kauffman and Martin. The discovery of iPSC enables the creation of pluripotent stem cell lines derived from living human patients replete with their complex undefined genetic backgrounds. For vascular biology, this advance allows for the creation of vascular endothelial cells with an unmodified genetic background on demand. While tremendously promising for basic and translational research and for novel therapeutic modalities, iPSC derived vascular endothelium remained relatively unexplored and certainly not adopted by the mainstream field of vascular biology. I posited that there may exist trepidation in adoption of such a novel platform due to the limited characterization of the fidelity, in particular in relation to the endothelia-specific functions, within iPSC derived endothelium. Thus, I began my this research constituting my Ph.D. dissertation in order to develop a protocol for the isolation of human

vascular endothelium from induced pluripotent stem cells, to systematically define the endothelia-specific cellular functions and to assess arterial and venous specification within iPSC endothelium. I approached this problem with scientific standards grounded from an endothelial cell biology laboratory, intending to hold a high bar of scrutiny when assaying the endothelial identity of this new artificial model system.

In this dissertation, I have generated vascular endothelium from human iPSC and characterized its functional repertoire. The methodology to differentiate pluripotent cells into vascular endothelium, by inducing differentiation of human iPSC colonies in embryoid body format, was reproducible and robust across several iPSC lines created with different technologies. I developed a methodology to dissociate these embryoid bodies, to isolate purified vascular endothelium and to establish the conditions to culture this endothelium *in vitro*. After isolation and culture, this endothelium displayed many characteristic molecular markers of vascular endothelium. Further, I observed vWF-positive functional Weibel-Palade Bodies that could rapidly exocytose to the cell surface in response to a physiological stimulus. These results suggest that iPSC derived endothelium exhibits a high molecular fidelity to primary human vascular endothelium.

Due to their promise as a novel *in vitro* model system for human vascular disease, I assessed whether iPSC endothelium is competent for the functional repertoire displayed by primary vascular endothelium which is known to be critical in several pathophysiological settings. Specifically, a fundamental function of the vascular endothelium is to acquire an activated phenotype and to participate in an inflammatory response by recruiting leukocytes to sites of inflammation. I documented that the iPSC endothelial cells were responsive to multiple pro-inflammatory stimuli (IL1β, TNFα, LPS and IFNγ) and capable of producing an activated pro-inflammatory phenotype that included expression of leukocyte adhesion molecules E-Selectin, ICAM-1 and VCAM-1, secretion of pro-inflammatory cytokines and support for human neutrophil and T lymphocyte rolling, arrest and transmigration. Another critical function of vascular endothelium is to serve as a dynamic barrier between blood and tissues. I

observed that iPSC endothelial cell monolayers display a dynamic barrier function capable of adjusting transendothelial permeability in response to a panel of physiological stimuli.

To document another specific function of vascular endothelium for which iPSC derived endothelial cells may provide a novel platform for *in vitro* disease modeling studies, I induced iPSC endothelium to acquire specific phenotypes previously implicated in vascular pathophysiology. I demonstrated that iPSC endothelial cells have the plasticity to adopt distinct flow-dependent phenotypes, namely atheroprotective and atheroprone phenotypes critical for atherosclerosis protection and susceptibility[80,86]. I observed that iPSC derived endothelial cells responded to atheroprotective shear stress by activating an atheroprotective gene expression program transcriptionally-mediated by the expression of the transcription factors *KLF2* and *KLF4.*The expression of these transcription factors has been shown to integrate the flow-mediated endothelial atheroprotective functional phenotype by regulating leukocyte adhesion, redox state and thrombotic function in cultured human EC[87-89,115-117]. The ability to direct the acquisition of flow-dependent functional and dysfunctional phenotypes using patient-specific endothelium should allow the study of specific genetic contributions to endothelial function and dysfunction in the context of human cardiovascular disease.

Another application of patient-specific endothelium capable of modeling functional and dysfunctional phenotypes is the evaluation of the effects of pharmacological agents on endothelial cells. I demonstrated that treatment of iPSC endothelial cells with simvastatin activated an atheroprotective gene expression program and down-regulated genes associated with an atheroprone phenotype. This suggests that iPSC endothelial cells may be a suitable surrogate to assess the effects of drugs on the endothelia of specific patients.

In this dissertation, I also sought to investigate the ability of iPSC derived vascular endothelium to adopt arterial and venous identities. One stimulus known to affect endothelial identity is biomechanical stimulation, in particular shear stress. I demonstrated that the application of shear stress derived from an arterial as opposed to that from a venous environment selectively induced the expression of a set of genes that are specifically enriched in arterial endothelium. Further, I observed that as iPSC differentiate within

embryoid bodies, they produce a heterogeneous population of endothelial cells. Based on upon previously published work identifying CXCR4 as an antigen able to distinguish arterial and venous endothelium, I observed that CXCR4 expression also distinguished endothelial subpopulations enriched for gene expression signatures indicating arterial or venous identity. Interestingly, the expression of a particular gene assayed, vWF, did not follow the anticipated expression pattern between the arterial and venous subsets, which are based on previous observations in mice and humans. As it appeared vWF was not differentially expressed in the arterial and venous iPSC derived endothelial cells based on genetic specification during development at least from the embryoid body system, I isolated these two subpopulations of endothelial cells from embryoid bodies to further investigate the mechanisms controlling the expression of vWF. I demonstrated that biomechanical stimulation specific to the arterial microenvironment repressed expression of vWF in both the arterial and venous endothelial cell subpopulations. This indicates that while many molecular aspects of arterial and venous endothelial identity are specified by genetic programs during differentiation, a subset of the features differentially present in arterial and venous endothelium are responsive to the divergent biomechanical stimuli present in arteries and veins. These results indicate that iPSC derived endothelium has the ability to acquire arterial and venous identities which may be critical for studying endothelial cell biology.

Collectively, these results advance human iPSC derived vascular endothelial cells as a novel experimental platform to study endothelial biology. This platform has tremendous versatility as it is 'personalizable;' able to produce vascular endothelium from any particular patient or genetic background. This dimension of patient-specificity adds a powerful dimension to the original conception of using isolated human endothelial cells for *in vitro* research. This advance is an enabling technology which is likely to promote new basic research and clinically translatable efforts as will be mentioned in the following section.

6.2 | Potential applications of personalized vascular endothelium

The discovery of induced pluripotent stem cells introduces the potential to derive all cell types bearing a particular patient's genetic background. This technology is an enabling platform that will empower new resources for drug discovery and personalized medicine. In this context, human iPSC have many advantages as a cellular substrate as they are: a) infinitely expandable in their pluripotent state, b) able to be cryopreserved, c) bear complete and precise genetic fidelity to the patient donor, d) immunocompatible with the patient donor, e) able to be generated from minimally invasive blood or skin samples, f) able to be genetically modified *ex vivo* and g) able to be produced without genome modification in xeno-free conditions. Human iPSC have been differentiated into many different cell types; however with poor functional characterization[118]. While iPSC have the biological potential to differentiate into every cell type, the salient challenge to widespread use of iPSC-derived tissues is the obstacle of robustly and reproducibly recreating embryonic cellular differentiation *in vitro* to produce mature functional cells. An important fraction of the research described in this dissertation seeks to provide a detailed functional characterization prerequisite for the widespread adoption of iPSC derived vascular endothelium for multiple research settings.

There is a significant need in both clinical and translational research settings for patient-specific vascular endothelial cells. In particular, the availability patient-specific vascular endothelial cells should enable: a) human cell-based models of genetic vascular disorders, b) personalized pharmacological efficacy and toxicity testing as vascular toxicity is a significant cause of drug candidate attrition[119], c) cell-based therapies for myocardial infarction and peripheral vascular disease, d) vascularization of engineered tissues, e) synthesis of vascular grafts and f) the maintenance of an anti-thrombotic surface for medical devices such as stents, hemodialysis machines and extracorporeal membrane oxygenation devices. Aside from grafts for vascular bypass, it is not currently possible to harvest sufficient endothelial cells from patients for the applications mentioned above. Of the many potential applications of patient-specific vascular endothelium, two appear to be the most promising and immediate. First, *in vitro* models

of drug induced vascular toxicity can be made to reduce attrition of experimental therapies and to help stratify patient populations. For comparison, an analogous to an effort to model drug induced cardiotoxicity with human iPSC has recently been reported[120]. The other promising, potentially immediate, and intellectually engaging is the possibility of using iPSC derived endothelial cells as a new model system to study the endothelial compartment in the context of genetic disorders. The following section outlines some of the potential and challenges that may be confronted in future translational disease modeling studies.

6.3 | Modeling the endothelial compartment within human genetic disorders

Employing iPSC to generate specific cell types to model a genetic disease presents several challenges. Here, one must create an iPSC line derived from tissue from patients presenting with a genetic disease of interest. These cells must be reprogrammed and differentiated into the cell type of interest. While this must be theoretically possible as the living patient is evidence that the disease-causing genetic mutation does not preclude pluripotency and development, there may be challenges efficiently recreating this process *in vitro* in the context of genetic abnormalities. Once a particular cell type with a given genetic mutation is produced, the mutation must be shown to be present in the cell type and further to have a functional consequence. There has been discussion as to the appropriate experimental control for such disease modeling, with the strongest candidate being iPSC lines genetically engineered to be isogenic to the disease cell line with the exception of a genetic alteration unraveling the original disease-causing genetic mutation. There has been some precedent for this genome editing approach with iPSC lines using zinc finger nuclease technology[121,122] and with transcription activator-like effector nucleases[123]. These technologies create two iPSC lines genetically identical with the exception of a point mutation corrected; hence, giving power to the specificity of a phenotype illustrated with the patient iPSC derived cells. However, there are many situations where such an approach may be inappropriate or not possible. More complex genetic abnormalities such as trisomies, copy number variations and other complex chromosomal disorders are not able to be corrected in this fashion. Moreover, one of the appealing strengths of iPSC in general is that the particular genetic mutations responsible for an observed phenotype known to be hereditary need not even be identified in order to produce iPSC lines and hence differentiated progeny bearing that unidentified mutation for further study. In such cases, other approaches to create appropriate control comparisons include generating iPSC lines from unafflicted siblings or parents which will bear genetic similarity to the disease model cell line. Alternatively, a strength-in-numbers approach is possible where iPSC lines are generated from many individuals both

healthy and afflicted with a particular disorder with the hope of capturing and perhaps averaging across different genetic variation.

Thus far, there are limited examples of modeling human cardiovascular diseases with iPSC. In particular, two groups have generated iPSC lines from patients with Hutchinson-Gilford Progeria Syndrome (HGPS), a genetic premature aging disorder that bears clinical resemblance to physiological aging[124]. Specifically, patients with HGPS develop advanced atherosclerosis, among other pathologies, at a young age that frequently gives rise to fatal myocardial infarctions and strokes. Previously, the study of cardiovascular disease within this rare disease population was restricted to studies on primary harvested dermal fibroblasts or histological study of cadaveric tissue. Both groups created HGPS iPSC lines and differentiated these cells into vascular smooth muscle cells to show modest increased senescence under several *in vitro* conditions suggesting a possible mechanism for the premature vascular disease observed in this disorder[125,126].

There are several other examples of genetic disorders where iPSC derived vascular endothelium may in the future provide a powerful model to evaluate endothelial function in cell based assays. For instance, the results in this dissertation demonstrate that iPSC display *in vitro* angiogenic potential and there are several genetic disorders wherein mutations occurring within angiogenic genes cause vascular malformations and disease[127]. Mutations within *VEGFR2* and *VEGFR3* genes have been linked to cases of infantile hemangioma, a vascular tumor[128]. Hereditary haemorrhagic telangiectasia is a disorder characterized by the presence of arteriovenous malformations, direction connections between arteries and veins eclipsing the capillary bed. In some instances, these malformations can lead to hypoxemia, stroke, heart failure and fatal hemorrhage. While mutations within genes involved in TGFβ signaling pathways such as *SMAD4*[129], *endoglin*[130] and *ALK1*[131], have been identified in patients with hereditary haemorrhagic telangiectasia, the pathological mechanisms responsible for the malformations remain unclear, complicated by the intricate TGFβ signaling network. Arterial tortuosity syndrome, a disorder marked by geometric abnormalities of medium and large arteries, has been shown to be caused by

mutation in a glucose transporter[132]. Mutations within several genes, *CCM1-3*, are associated with dilated capillaries and saccular vascular structures in the brain, termed cerebral cavernous malformations[133,134]. There are numerous other examples of vascular malformations with genetic origins[1]. In all these instances, the creation of a human genetic model through induced pluripotent stem cells coupled with assessment of angiogenic potential may elucidate the functional consequences and molecular mechanisms behind these vascular malformations.

In response to pro-inflammatory stimuli, the vascular endothelium enters an activated phenotype characterized by the secretion of pro-inflammatory cytokines and chemoattractants and presentation of intercellular adhesion molecules to its surface[70,71,72,73]. This coordinated response can promote the interaction of monocytes, neutrophils, and T cells and the vascular wall mediating many inflammatory processes. These elements of endothelial activation, also observed within iPSC derived endothelium as observed above, may provide a new *in vitro* model of inflammatory disease. This leukocyte-endothelial interaction has significant consequences for the defense against pathogens, atherosclerosis[135,136] and autoimmune disorders such as systemic lupus erythematous[137], Kawasaki disease[138], scleroderma[139]. While many of the specific mediators, chemokines and adhesion molecules, have been identified, the signaling mechanisms responsible for these interactions are not fully understood, especially in the context of cardiovascular, autoimmune diseases and rare diseases. Frequently, the genetic determinants of the endothelial dysfunction in these disorders have not been identified. In this case, iPSC derived endothelium presents a system of capturing the genetics of complex human disease without even having to identify the particular pathological genetic variation. The interaction of leukocytes and endothelium has been studied *in vitro* under a variety of inflammatory contexts. Here, the biophysical interaction of the leukocyte, rolling, slow rolling, arrest, and transmigration, occur amidst specific hemodynamic conditions in the vasculature that can be recreated with *in vitro* flow systems[140,141,142]. My results suggest that iPSC derived endothelium may be an appropriate model system to study the interaction of leukocytes and patient-specific endothelium as iPSC endothelial cells were able to adopt an activated phenotype and support leukocyte transmigration.

An essential function of the vascular endothelium is to maintain a dynamic and selectively permeable barrier regulating the transport of fluid and macromolecules between the circulation and the interstitial space. The dynamism of this barrier function is regulated by a variety of soluble factors, cell surface receptors, signaling molecules, cytoskeletal and other structural proteins[143,76]. Vascular endothelial permeability is critical for maintaining vascular homeostasis and for access of pharmacological agents to cross the specialized blood brain barrier. Further, endothelial permeability contributes to inflammatory processes[144], dysfunctional vasculature near solid tumors[145] as well as in chronic disease states. In particular, the advanced glycation end products present in diabetic vasculature have been shown to influence endothelial barrier function resulting in vascular hyperpermeability[146,147] with several molecular mediators identified[148]. The complex effects of these pathological settings on endothelial permeability can be assayed *in vitro* most commonly either through detection of trans-cellular transport of fluorescently labeled macromolecules such as dextran through dual chambered culture devices, or by measurement of electrical impedance between microelectrodes across endothelial monolayers[149,150]. These assays are highly quantitative and are able to produce time dependent permeability data able to discern transient effects. Miniaturization and parallelization of these assays, both fluorescent-based macromolecule transport[151] and commercially available 96-well platforms and culture plates for measuring transendothelial electrical resistance, permit screening assays to use endothelial permeability as a direct functional output. This dimension of vascular homeostasis represents another area where patient-specific iPSC derived vascular endothelium may contribute to new translational research.

It may be that by employing human iPSC derived cells with appropriate genetic background, recreating precise physiological environments and by using cell based assays of cellular function, it is possible to more faithfully reproduce pathophysiological processes *in vitro* and offer a more relevant human experimental platform for drug discovery. Above, I introduced several vascular endothelial functions that the pharmaceutical industry has been able to mimic and miniaturize for at least medium throughput experiments which in conjunction with new patient-specific iPSC derived vascular endothelium may prove useful for the discovery of new cardiovascular therapies and drug targets. The

technology exists to further increase parallelization should industrial rather than academic laboratories become more interested in their use. This may be of particular importance in research on orphan and rare diseases in which little is known about the molecular basis of the disease and where primary tissue is unavailable for study.

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